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Analysis of Met-Form Haemoglobins in Human Erythrocytes of Normal Adults and of a Patient with Hereditary Methaemoglobinaemia due to Deficiency of NADH–Cytochrome b_5 Reductase

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Isoelectric-focusing analysis on an Ampholine/polyacrylamide-gel plate revealed that met-form haemoglobins are present as half-oxidized haemoglobins such as the $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ forms rather than as methaemoglobin in the erythrocytes of normal human adults and also of a patient with hereditary methaemoglobinaemia due to deficiency of NADH–cytochrome b_5 reductase.

It seems to be generally accepted that the oxidized haemoglobin, which represents less than 1% of the total haemoglobins in normal human erythrocytes, is methaemoglobin (Bodansky, 1951). However, analytical investigations into whether the met-form haemoglobin is fully oxidized or partially oxidized have not been reported. Previously we showed that met-form haemoglobin in glucose-depleted erythrocytes is not MetHb, but half-oxidized haemoglobin (Tomoda *et al.*, 1978*a*). This finding suggests that fully oxidized haemoglobin (methaemoglobin) might be absent in human erythrocytes even under physiological conditions. To study the possibility further, we carried out the identification of the met-form haemoglobins in intact human erythrocytes of normal subjects and also from a patient with hereditary methaemoglobinaemia due to deficiency of NADH–cytochrome b_5 reductase, by using isoelectric-focusing analysis.

As a result, it was found that these contain half-oxidized haemoglobins such as the $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ forms rather than MetHb as met-form haemoglobins. On the basis of the results, the mechanism of haemoglobin oxidation in human erythrocytes is discussed.

Experimental

Blood samples (5ml) were freshly obtained without anticoagulant from 20 normal human adults (with their informed consent). Clotting of the blood samples was not observed, since silicone-coated injectors and glass tubes were used for collecting the blood and the samples were stood in an ice bath. The samples were centrifuged at 10000rev./min ($r_{av.}$ 7.2cm) for 1min within 30min. After removal of the plasma, the erythrocytes were suspended in 0.9% NaCl solution and centrifuged at 10000rev./min for 1min. The erythrocytes obtained by this procedure were haemolysed by the addition of 5vol. of ice-cold distilled water. The haemolysates were further centrifuged at 10000rev./min for 20min to remove 'ghosts', and used for the experiments.

The heparinized blood of a patient with hereditary methaemoglobinaemia due to deficiency of NADH–cytochrome b_5 reductase was also treated by the same procedure as stated above within 4h of drawing the sample, and used for the experiments. [The ferricyanide reductase and NADH–cytochrome b_5 reductase activities in the haemolysate, which were measured by the methods of Hegesh & Avron (1967) and Sugita *et al.* (1971) respectively, were extremely low.]

Concentrations of met-form haemoglobins were determined as described by Evelyn & Malloy (1938). The contents of met-form haemoglobin of normal haemolysates (20 cases) were 0.3–0.6% of those of total haemoglobin. The met-form content of the haemolysate of the patient with methaemoglobinaemia was 17%.

The haemolysates were applied to an Ampholine/polyacrylamide-gel plate (3cm×11cm; pH3.5–9.5; LKB Produkter, Bromma 1, Sweden) and isoelectric focusing was performed at 4°C for 1.5h (settled at initial current 6mA, final voltage 1200V and constant 6W). Then the gel plate was fixed with solutions containing 0.7M-trichloroacetic acid, 0.14M-sulphosalicylate and 7M-methanol and scanned at 630nm with a Gilford 2400-S spectrometer with a gel scanner. In each fraction the ratio of haemoglobin

Abbreviations used: HbA, haemoglobin A; MetHb, methaemoglobin. Throughout this paper the haemoglobins are, for convenience, referred to as though they were the unoxygenated forms.

derivatives to total haemoglobins was determined by cutting out and weighing the chart paper.

Results

Distribution of met-form haemoglobin in normal erythrocytes

Fig 1(*a*) shows the isoelectric-focusing pattern of both normal and ferricyanide-treated haemolysates on the Ampholine/polyacrylamide-gel plate. In the haemolysates that were partially oxidized by ferricyanide, the intermediate bands IB_I and IB_{II} (see Fig. 1) and MetHb were found in addition to HbA. However, in a normal haemolysate, MetHb was not found, but IB_I and IB_{II} were detected as met-form haemoglobins. Since we showed that IB_I and IB_{II} are $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ forms of haemoglobin respectively (Tomoda *et al.*, 1978*b*), it is evident that met-form haemoglobins in normal erythrocytes are not MetHb, whose subunits are fully oxidized, but are half-oxidized haemoglobin forms such as $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$.

The electrophoretic pattern was further analysed by gel-scanning as shown in Fig. 1(*b*). From the results, the percentage of each component to total haemoglobins was determined by cutting out and weighing the portion of chart paper corresponding to each fraction; HbA, haemoglobin F, haemoglobin $(\alpha^{2+}\beta^{3+})_2+(\alpha^{3+}\beta^{2+})_2$ and haemoglobin A_2 were 93.5, 0.65, 0.85 and 5% respectively of total haemoglobin. Since the ferric-haem content was 0.4% in the haemolysate, it was expected that half-oxidized haemoglobins would be present as 0.8% of total haemoglobin if MetHb was not present. The results obtained from Fig. 1(*b*) (0.85%) are in good agreement with this expectation. As to other cases of normal haemolysates, the same results were obtained.

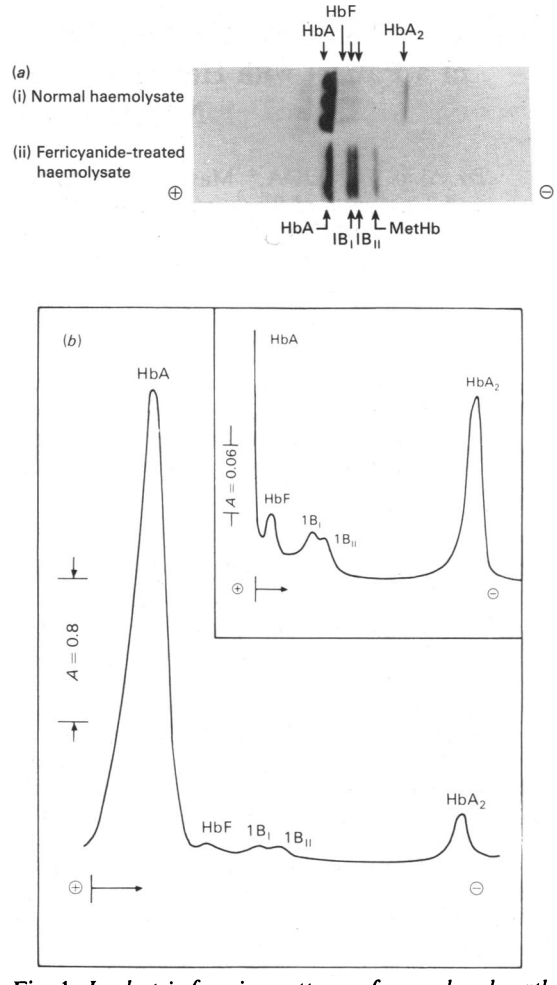


Fig. 1. *Isoelectric-focusing patterns of normal and partly oxidized haemolysates*

(*a*) Protein band pattern of normal haemolysate (i) compared with that of haemolysate partially oxidized with ferricyanide (ii) (2mol/mol of haemoglobin tetramer). (*b*) Gel-scanning pattern of normal haemolysate performed at 630nm on a Gilford 2400-S spectrometer with a gel scanner. The inset shows a section of the main pattern on a different scale. Abbreviations used: HbA, oxyhaemoglobin; IB_I, intermediate band I; IB_{II}, intermediate band II; HbA_2, oxygenated haemoglobin A_2; MetHb, methaemoglobin; HbF, oxygenated haemoglobin F.

Distribution of met-form haemoglobins in the erythrocytes of a patient with heriditary methaemoglobinaemia

It was shown that the contents of MetHb are increased in the erythrocytes of patients with hereditary methaemoglobinemia due to deficiency of NADH–cytochrome b_5 reductase. However, it remained obscure whether the met-form haemoglobins in the haemolysate of such a patient are fully oxidized or partially oxidized forms, since the MetHb contents were conventionally measured by the method of Evelyn & Malloy (1938) as total ferric haem.

We analysed met-form haemoglobins in haemolysate of the methaemoglobinaemia patient by isoelectric focusing on an Ampholine/polyacrylamide-gel plate (Fig. 2*a*). As a result, there appeared the five main bands, which corresponded to HbA, form $(\alpha^{2+}\beta^{3+})_2$, form $(\alpha^{3+}\beta^{2+})_2$, MetHb and haemoglobin A_2 from the the anode, and the intensities of MetHb and haemoglobin A_2 were relatively small.

The percentage of these components in relation to total haemoglobins is easily determined by scanning the fixed gels (Fig. 2*b*). HbA, form $(\alpha^{2+}\beta^{3+})_2$, form $(\alpha^{3+}\beta^{2+})_2$, MetHb and haemoglobin A_2 were determined to be 60.6, 12.7, 18.5, 4.4 and 3.8%

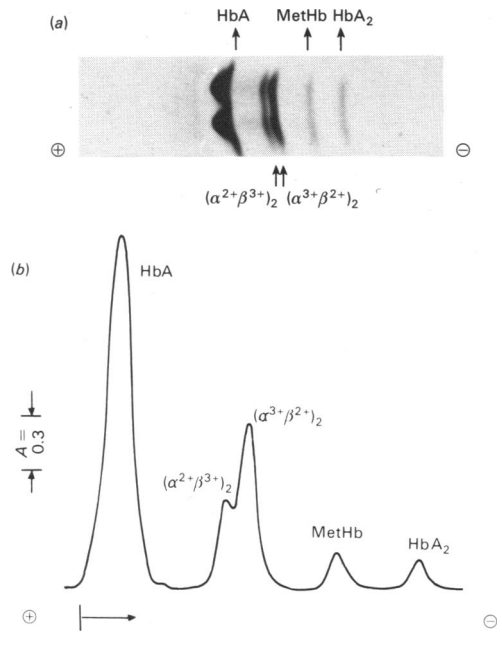
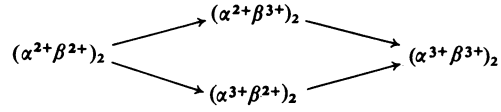


Fig. 2. *Isoelectric-focusing patterns of a methaemoglobinaemia-patient's haemolysate*
(*a*) Protein band pattern; (*b*) gel-scanning pattern.

respectively. The summation of ferric haem of form $(\alpha^{2+}\beta^{3+})_2$, form $(\alpha^{3+}\beta^{2+})_2$ and MetHb (20%) was in good agreement with the value obtained by the method of Evelyn & Malloy (1938), namely 17%.

Discussion

Since MetHb was shown to be formed during the oxidation of isolated haemoglobin (Kikuchi *et al.*, 1955; Mansouri & Winterhalter, 1973), it seems to be generally believed that MetHb is also produced in human erythrocytes. However, present results show that half-oxidized haemoglobins such as the $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$ forms are present rather than MetHb as met-form haemoglobins in the erythrocytes under physiological conditions. The half-oxidized haemoglobins are predominant even in the methaemoglobinaemia-patient's erythrocytes, where met-form haemoglobins are known to be increased to a large extent (Gibson, 1948; Scott & Hoskins, 1958). From these results, the course of haemoglobin oxidation in human erythrocytes can be envisaged as follows:



MetHb was not found in normal erythrocytes and was not accumulated to any great extent in the methaemoglobinaemia-patient's erythrocytes. These results suggest two possibilities: one is that the rate constants of oxidation of half-oxidized haemoglobins, to MetHb are very small compared with that of HbA to half-oxidized haemoglobin; another is that the quantity of MetHb formed from half-oxidized haemoglobin is small simply because the half-oxidized haemoglobins as substrates do not accumulate so much. It would be necessary to study the kinetic behaviour of haemoglobin oxidation to distinguish between these two possibilities.

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