Differential effect of detergents on the alkaline denaturation of haemoglobin in maternal and fetal blood, with particular reference to Triton X-100

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SUMMARY In this investigation, the alkaline denaturation of haemoglobin in the blood of pregnant women and in cord blood obtained from newborn infants was followed by measuring the increase in absorbance at 375 nm. As expected, in the absence of detergent, the haemoglobin of cord blood was much more resistant to alkaline denaturation than that of maternal blood. However, in the presence of Triton X-100, a non-ionic detergent, the sensitivity of fetal haemoglobin to alkali was comparable to that of adult haemoglobin. Similar results were obtained using the non-ionic detergents, Brij-35, Tween 80 and Nonidet P40, but the anionic detergent, sodium deoxycholate, was apparently without effect. These findings form the basis of a rapid and sensitive method for discriminating between maternal and fetal blood in biological specimens.

In the presence of alkali, adult and fetal haemoglobin are both denatured yielding alkaline hematin.¹ However, fetal haemoglobin is much more resistant to alkaline denaturation than adult haemoglobin, and this difference forms the basis of classical methods for determining the relative proportions of fetal and adult haemoglobin in blood.¹ At 11-12 wk gestation, all the haemoglobin in the blood of the fetus is of the alkali-resistant fetal type.² Blood collected from the umbilical cord at the time of delivery of the infant also contains a substantial proportion of fetal haemoglobin.¹ At 28-34 wk gestation, the fetal haemoglobin content is consistently about 90%,^{2,3} while a mean value of about 75% fetal haemoglobin, with individual values ranging from 50 to 88%, has been reported for cord blood collected at term.^{2,4} In contrast, virtually all the haemoglobin in normal adult blood is alkali-sensitive.⁵

In this paper, differences in the effect of detergents on the alkaline denaturation of haemoglobin in maternal and cord blood are described. This investigation was prompted by our requirement for a rapid and sensitive method for determining whether blood, present as a contaminant in some samples of amniotic fluid used for the evaluation of fetal lung maturity,⁶ is of maternal or fetal origin.

Material and methods

Blood was collected, in heparinised tubes, from pregnant women near term and from the umbilical cord of both term and preterm newborn infants. Amniotic fluid was obtained by trans-abdominal amniocentesis as part of routine management of complicated pregnancies.

The rate of denaturation of haemoglobin in the presence of alkali was determined as follows: blood was diluted 1/10 with normal saline before use. The reaction mixture contained diluted blood (50 μ l, unless otherwise specified) and Na₂CO₃ (0.1 g/l) to a final volume of 3 ml. After measuring the initial absorbance at 375 nm, 12-25 μ l standardised 5 mol/l NaOH was added with rapid mixing and the absorbance was recorded for a further 5 min. A Pye Unicam SP8-100 Spectrophotometer was used and all reactions were routinely carried out at 25°C.

Appropriate volumes of solutions of the following detergents (20 g/l, unless otherwise specified) were added to the reaction mixture, before or after the addition of NaOH, as required: Triton X-100 (Rohm and Haas), sodium deoxycholate (E Merck), sodium dodecyl sulphate and Brij-35 (Sigma), Tween 80 (Chemical Materials), Nonidet P40 (Shell Chemicals) and cetyltrimethylammonium bromide (British Drug Houses).

Total haemoglobin was determined by the cyanmethaemoglobin method (Sigma).

OPTIMISED PROCEDURE FOR DISCRIMINATING BETWEEN MATERNAL AND FETAL BLOOD

Follow the procedure described above, using 25 μ l 5 mol/l NaOH. Measure the absorbance at 375 nm for 1 min after the addition of NaOH, then add 25 μ l Triton X-100 (40 g/l) with immediate thorough mixing and measure the absorbance for a further 2 min.

Results

Denaturation of haemoglobin by NaOH was accompanied by a marked increase in absorbance between 340 and 390 nm. The maximum change in absorbance occurred at 372 nm for both maternal and cord blood, in the absence of detergent, and at 377 nm in the presence of Triton X-100.

In this investigation, assay conditions were deliberately chosen to highlight the well-known difference in the effect of alkali on haemoglobin in maternal and cord blood, in the absence of detergent (Fig. 1). In the presence of 40 mmol/l NaOH, the denaturation of haemoglobin in maternal blood was complete within 1 min (Fig. 1b), whereas for cord blood, the reaction took about 2 h to approach completion (data not shown). However, when Triton X-100 was included in the reaction mixture, the difference in the sensitivity of the haemoglobin in maternal and cord blood to alkali was largely abolished (Fig. 1); haemoglobin from both sources was denatured rapidly, yielding approximately the same change in absorbance per milligram of haemoglobin in each case.

The effect of varying the concentration of Triton X-100 in the reaction mixture is shown in Fig. 2, which also serves to illustrate the between-sample reproducibility of the phenomenon.

Other non-ionic detergents tested, Brij-35, Tween 80 and Nonidet P40, also behaved like Triton X-100 in that they tended to abolish the difference in the response of maternal and cord blood to alkali, the least effective (on a weight for weight basis) being Tween 80 (Table 1).

In contrast, sodium deoxycholate, a naturally-occurring anionic detergent, appeared to be without effect (Table 1). However, the addition of another anionic detergent, sodium dodecyl sulphate, to either cord or maternal blood in itself caused a time-dependent increase in absorbance, with a subsequent very rapid increase on the addition of NaOH (data not shown). The cationic detergent, cetyltrimethylammonium bromide, appeared to cause immediate denaturation of haemoglobin in both maternal and cord blood in the absence of NaOH.

The overall result was the same, whether Triton X-100 was added before the alkali or one minute later (Fig. 3). In this experiment, for samples treated with Triton X-100 after the alkali (Fig. 3, solid lines), the procedure was identical to that described as the "Optimised procedure" in the Material and methods section. Under these conditions, only a short reaction time is required to give good discrimination between maternal and cord blood.

In the optimised procedure, the results were only

Fig. 1 Effect of Triton X-100 on the denaturation of haemoglobin by alkali. NaOH was added at zero time to reaction mixtures containing maternal blood (solid lines) or cord blood (broken lines) and the resulting change in absorbance at 375 nm (ΔA) was measured. The final concentration of NaOH was 20 mmol/l (a) or 40 mmol/l (b). The reaction was carried out in the absence (○) or presence (●) of Triton X-100, 0.3 g/l final concentration.

Fig. 2 Effect of varying Triton X-100 on the change in absorbance measured 4 min after the addition of NaOH (30 mmol/l, final concentration). Each point represents the mean value ($\pm$ SD) for three samples of cord blood ($\bullet$), one collected at 33 wk and two at 40 wk gestation, and three samples of maternal blood ($\circ$).

slightly affected by varying the concentration of blood; the shape of the curve given by haemoglobin in maternal blood was always distinctly different from that in cord blood, as indicated by the ratio of the absorbances at 3 min and 1 min (Table 2). Varying the temperature of the assay from 20 to 30°C or including 200 μ l of blood-free amniotic fluid in the reaction mixture also had a negligible effect on the results (data not shown). The present procedure is

Table 1 Comparison of the effects of various detergents

Detergent*	Change in absorbance/mg Hb†			
	Maternal blood		Cord blood	
	1 min	4 min	1 min	4 min
None	0.55	0.53	0.07	0.11
Triton X-100	0.42	0.44	0.37	0.45
Brij-35	0.40	0.42	0.37	0.43
Tween 80	0.45	0.46	0.16	0.28
Nonidet P40	0.43	0.44	0.42	0.46
Deoxycholate	0.54	0.52	0.07	0.11

*Detergent or distilled water (30 μ l) was added to the reaction mixture before measuring the initial absorbance.

†NaOH was added at zero time (final concentration, 40 mmol/l).

Table 2 Effect of varying blood concentration on the response of maternal and cord blood to NaOH and Triton X-100

Volume of dil blood (μ l)	Change in absorbance/mg Hb*			
	Maternal blood		Cord blood	
	1 min	3 min/1 min	1 min	3 min/1 min
25	0.55	0.75	0.06	6.7
50	0.52	0.79	0.06	7.1
75	0.49	0.82	0.05	7.6
100	0.46	0.85	0.05	7.8

*The reaction was carried out according to the optimised procedure (see Material and methods). The final concentration of blood was 0.8–3.3 ml/l.

therefore relatively independent of temperature, within the limits prevailing in most laboratories, and can be used to assess blood-stained samples of amniotic fluid.

Fig. 3 Effect on the denaturation of haemoglobin of adding Triton X-100 before or after the alkali. The reaction mixture contained (a) maternal blood or (b) cord blood (33 wk gestation); 25 μ l NaOH was added at zero time and 25 μ l Triton X-100 (40 g/l) was added either before (broken lines), or 1 min after the NaOH with immediate mixing (solid lines).

Discussion

In other investigations in which the conversion of haemoglobin to alkaline hematin has been followed by direct spectrophotometric analysis, the increase in absorbance at 630 nm or the decrease in absorbance at 540–580 nm has been measured.¹ Measuring the corresponding increase in absorbance at 375 nm gives a 7- or 3.5-fold increase in sensitivity, compared with measuring at 630 or 540 nm, respectively.

The shape of the curve given by either maternal or cord blood in response to the consecutive addition of alkali and Triton X-100 (as in the optimised procedure) is quite distinctive and the difference between cord and maternal blood is obvious within 2 min of adding the alkali (Fig. 3). Under these conditions, the increase in absorbance during the first minute (that is, in the absence of detergent) presumably represents rapid denaturation of adult haemoglobin with a variable, but usually small, contribution due to the slow denaturation of any fetal haemoglobin in the sample. On the addition of Triton X-100, the observed absorbance change presumably represents the net effect of an increase in absorbance, due to the augmentation of denaturation of any fetal haemoglobin in the sample, and a decrease in absorbance due to the interaction of the detergent with the denatured haemoglobin.

Using the optimised procedure described in this paper, it is not necessary to know the haemoglobin content of the sample in order to distinguish maternal from fetal blood. In contrast, in the absence of detergent, discrimination between maternal and cord blood is not always possible unless the change in absorbance is related back to the haemoglobin content of the sample or the reaction is allowed to proceed for a long time. For example, in Fig. 1, the difference in the curves given by maternal and cord blood in the absence of detergent is quite obvious within 5 min, but this is largely due to the fact that absorbance changes have been expressed per mg haemoglobin. This feature of the method is especially important when haemoglobin concentrations are low, a frequent occurrence in our laboratory where blood in 160 μ l samples of lightly-contaminated amniotic fluid is assessed routinely. Using the optimised procedure, clearcut discrimination between blood of fetal and maternal origin is possible even when the reaction mixture contains as little as 0.1 ml blood per litre, giving an overall absorbance change at 3 min of about 0.02.

While the proportion of fetal haemoglobin in cord blood is generally high, it may be as low as 50% in some infants delivered at term.^{2,4} On the assumption that the samples of maternal and preterm cord blood

used to derive the data in Table 2 contained 100% adult haemoglobin and 10% adult-90% fetal haemoglobin, respectively,²⁻⁴ it can be calculated that a sample of blood containing 50% fetal-50% adult haemoglobin would give a 3 min/l min absorbance ratio of about 1.5, using the optimised procedure. This is appreciably higher than the 3 min/l min ratio given by maternal blood (Table 2). Therefore the present method can be used to determine whether blood present in an amniotic fluid sample is of fetal or maternal origin, even when the fetal blood contains a minimal proportion of fetal haemoglobin.

The present procedure meets our need for a rapid, simple and sensitive method for assessing blood in amniotic fluid and, for this specific application, is preferable to existing methods. These include alkali-denaturation methods involving spectrophotometry with or without precipitation of the alkaline hematin with ammonium sulphate^{1,5} and histological methods—for example, Kleihauer *et al.*⁷ More sophisticated techniques, also available, include electrophoresis, radial immunodiffusion and high performance liquid chromatography. The present method offers a simple alternative to existing methods in any application which requires only discrimination between blood of fetal or maternal origin. It is especially useful when only a small amount of blood is present in the sample. With further development, this approach could also be used to obtain more quantitative information about the relative proportions of adult and fetal haemoglobin present in the blood.

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