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A Comparison of Erythrocyte Glutathione *S*-Transferase Activity from Human Foetuses and Adults

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Glutathione *S*-transferase activity was measured in partially purified haemolysates of erythrocytes from human foetuses and adults. Enzyme activity was present in erythrocytes obtained between 12 and 40 weeks of gestation. The catalytic properties of the enzyme from foetal cells were similar to those of the enzyme from adult erythrocytes, indicating that probably only one form of the erythrocyte enzyme exists throughout foetal and adult life.

The glutathione *S*-transferases are a group of dimeric enzymes of mol.wt. about 45000 that are found in a variety of organisms and tissues (Jakoby & Keen, 1977; Jakoby, 1978). The purified enzymes from human and rat liver have been extensively studied and, although their precise function has not been defined exactly, it appears that they are involved in the detoxification of both endogenous and exogenous compounds. Detoxification may occur by one of three mechanisms: firstly, non-covalent binding of non-substrate anions, including bilirubin and Indocyanine Green, secondly, covalent binding of toxic compounds such as 1-chloro-2,4-dinitrobenzene, and thirdly, catalysis of reactions in which GSH is conjugated with a wide range of electrophiles to form a thioether (Jakoby & Keen, 1977; Jakoby, 1978). Because of this lack of substrate specificity, the glutathione *S*-transferases have been given a variety of names, including glutathione *S*-alkenetransferases and glutathione *S*-epoxidetransferases. Although the intra-uterine development of the different proteins in this group of enzymes has not been formally studied in man, both glutathione *S*-alkenetransferase (Chasseaud, 1973) and glutathione *S*-epoxidetransferase (Juchau & Namkung, 1974) activities have been reported in 100000 *g* supernatants of livers from human foetuses (Dutton, 1978; Chasseaud, 1979).

Marcus *et al.* (1978) described a glutathione *S*-transferase from human erythrocytes. Unlike the glutathione *S*-transferases from human liver the erythrocyte enzyme has an acidic isoelectric point (pI 4.5). The amino acid composition of the erythrocyte enzyme is different from that of the liver enzymes, indicating that a separate gene is responsible for the synthesis of the glutathione *S*-transferase protein in human erythrocytes (Marcus *et al.*, 1978). We now describe the time-course of appearance of this enzyme during intra-uterine life and compare the catalytic properties of the foetal enzyme with those of the enzyme from erythrocytes of adult humans.

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Abbreviation used: GSH, reduced glutathione.

Materials and Methods

Chemicals

Analytical-grade 1-chloro-2,4-dinitrobenzene was obtained from BDH Chemicals, Poole, Dorset, U.K. CM-Sephadex C-50, DEAE-Sephadex A-50 and Sephadex G-75 were purchased from Pharmacia Fine Chemicals, London W5 5SS, U.K. All dialysis steps were performed at 4°C by using Spectrapor dialysis membrane (mol.wt. cut-off 12000–14000; Spectrum Medical Industries, Los Angeles, CA, U.S.A.).

Collection of blood samples and preparation of haemolysate

Blood (20 ml) was obtained from adults from the antecubital vein, heparinized and centrifuged (10 min, 10°C, 1000 *g*), and the plasma and buffy layer lying on top of the erythrocytes were removed. The remaining erythrocytes (about 10 ml) were resuspended in 50 ml of NaCl (154 mM) and re-centrifuged (15 min, 2°C, 1500 *g*). The cells were resuspended and re-centrifuged a further four times. The washed erythrocytes were then haemolysed by suspension in 2 vol. of distilled water, followed by freezing at −20°C. After gentle thawing, the solution was centrifuged (1 h, 2°C, 100000 *g*) and

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the supernatant was decanted. This supernatant is referred to as the haemolysate.

Blood from newborns (25–40 weeks gestation) was obtained either by venepuncture at the placental end of the separated umbilical cord (10ml) immediately after birth, or through an indwelling cannula in the umbilical artery (2ml). Blood from newborns was taken for routine clinical chemical assessment. Blood (0.5–2ml) from aborted foetuses (12–18 weeks gestation) was taken by cardiac puncture. Haemolysates were prepared from foetal blood as described above. Permission to take blood was obtained from the Ethics Committee of the Simpson Memorial Maternity Pavilion. In the conceptus studied, development was appropriate for gestational age.

Analytical methods

Glutathione *S*-transferase activity was assayed at 25°C by monitoring the increase in A_{340} by using, except where indicated, GSH (1mM) and 1-chloro-2,4-dinitrobenzene (1mM) as substrates (Habig *et al.*, 1974). The reaction volume was 1.25ml. Reaction rates were corrected for the small rate (usually less than 5% of the total rate) of non-enzyme-catalysed conjugation of GSH with 1-chloro-2,4-dinitrobenzene. Na^+ was measured by flame photometry (Strange *et al.*, 1977) and protein by the biuret method (Herbert *et al.*, 1971), with bovine serum albumin as the standard.

CM-Sephadex chromatography

Before assay, the glutathione *S*-transferase activity in foetal, neonatal and adult erythrocytes was partially purified by elution from CM-Sephadex. Haemolysate was first dialysed against 2 litres of sodium phosphate buffer (10mM-phosphate, pH6.45) and eluted (4°C, 21ml/h) from columns of CM-Sephadex. Haemolysate from newborns and adults (about 10ml; 150mg of protein/ml) was eluted from columns (2.2cm × 33cm) which were equilibrated with sodium phosphate buffer (10mM, pH6.45). The fraction volume was 4ml. The NaCl gradient, started after 80ml had been eluted, was established by adding (21ml/h), with mixing, sodium phosphate buffer (10mM, pH6.45) containing NaCl (500mM) to the 100ml of sodium phosphate (10mM, pH6.45) in the reservoir supplying the column. Haemolysate from aborted foetuses (4ml; 70mg of protein/ml) was eluted from smaller columns of CM-Sephadex (2cm × 15cm); the fraction volume was 2ml. The NaCl gradient, started after 40ml had been eluted, was established as described above.

Glutathione S-transferase activity during intra-uterine life

Glutathione *S*-transferase activity was eluted from CM-Sephadex as a single peak of enzyme activity; no activity was retained by the ion-exchanger. The fractions containing enzyme activity (haemolysates from adult and newborns, elution volumes 40–60ml; haemolysates from aborted foetuses, elution volumes 22–30ml) were combined and portions (50μl; about 1.5mg of protein/ml) used in the enzyme-assay mixture. Enzyme activity was measured in partially purified preparations obtained from 11 foetuses and newborns (12–40 weeks gestation).

Comparison of the glutathione S-transferase from adult and foetal erythrocytes

The combined enzyme-containing fractions from CM-Sephadex were also used to compare the catalytic properties of the adult and foetal enzymes. For this comparison, erythrocytes were obtained from two adults (males, 19 and 21 years), three newborns (36–40 weeks gestation) and two aborted foetuses (15 and 18 weeks gestation). Enzyme assays were performed in sodium phosphate buffer (100mM) and, except where indicated, at pH6.5 and 25°C.

Determination of molecular weight of glutathione S-transferase in haemolysate

Portions of haemolysate from foetal and adult erythrocytes (5ml; about 100mg of protein/ml) were eluted (4°C, 25ml/h) from a column of Sephadex G-75 (2.2cm × 30cm) with sodium phosphate buffer (100mM; pH7.5). Fraction volume was 4ml, void volume (Blue Dextran) was 70ml and the elution volume of Na^+ was 220ml. The column was calibrated for determination of protein molecular weight by measuring the elution volumes of bovine serum albumin (mol.wt. 67000), haemoglobin (mol.wt. 65000), intestinal glutathione *S*-transferase (mol.wt. 47000), ovalbumin (mol.wt. 43000) and ribonuclease (mol.wt. 12500). The molecular weight of the glutathione *S*-transferases in the different haemolysate preparations were calculated from the linear plot of log (molecular weight) against the elution volume of the protein standards.

Discontinuous polyacrylamide-gel electrophoresis

The subunit composition of the glutathione *S*-transferase in haemolysate was studied by discontinuous sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Electrophoresis was performed in the presence of 0.1% sodium dodecyl sulphate in vertical slab gels (0.075cm × 12cm × 20cm) as described by Laemmli (1970) and Hayes *et al.* (1979).

Before electrophoresis, the glutathione *S*-transferase in haemolysate was partially purified. Samples of haemolysate from foetal (15 weeks gestation), neonatal (40 weeks gestation) and adult blood were eluted from CM-Sephadex as described above. Portions (10ml) of the combined glutathione *S*-transferase-containing fractions were eluted from

Sephadex G-75, and the three fractions from the gel-exclusion column which contained the maximum enzyme activity (elution volume 97–104 ml) were combined and concentrated to about 2 ml by dialysis (4°C; 1 h) against poly(ethylene glycol). Portions of this solution were studied by electrophoresis. The gels were calibrated for determination of protein molecular weight by measuring the migration distances of the Ya (mol.wt. 22000), Yb (mol.wt. 23500) and Yc (mol.wt. 25000) monomers of purified preparations of ligandin and glutathione *S*-transferase B from rat liver cytosol (Hayes *et al.*, 1979).

DEAE-Sephadex chromatography

To examine the possibility that more than one enzyme with glutathione *S*-transferase activity is present in the erythrocyte haemolysates, portions of haemolysate were eluted from columns of DEAE-Sephadex. Haemolysate was first dialysed (4°C, 15 h) against 2 litres of Tris/HCl buffer (10 mM, pH 8.6). Dialysed solution (about 100 mg of protein/ml; from adult and newborn 5 ml, and from aborted foetus 2 ml) was eluted (4°C, 21 ml/h) from a column of DEAE-Sephadex (2.2 cm × 36 cm) equilibrated with the Tris/HCl buffer. The NaCl gradient, started after 80 ml had been eluted, was established by adding the Tris/HCl buffer containing NaCl (400 mM) to 100 ml of the Tris/HCl buffer in the reservoir supplying the column.

Glutathione S-transferase activity in the haemolysate 100000 g pellet

Blood (10 ml) from adult and neonatal subjects was centrifuged (10 min, 2°C, 1500 *g*) and the plasma removed. The erythrocytes (5 ml) were lysed by the addition of distilled water (5 ml), frozen (30 min, −20°C) and, after thawing, centrifuged (100000 *g*, 2°C, 40 min). The supernatant was decanted and the pellet washed twice with NaCl (154 mM) and once with sodium phosphate buffer (100 mM; pH 7.5). The pellet was then gently resuspended in 1 ml of sodium phosphate buffer (100 mM; pH 7.5) and portions (50 μl; 2.0–5.0 mg of protein/ml) were incubated (20°C; 30 min) in sodium phosphate buffer (200 μl; 100 mM; pH 7.5) with or without iodoacetamide (10 mM). After incubation, portions (50 μl) of the suspension were assayed for glutathione *S*-transferase activity, with GSH (5 mM) and 1-chloro-2,4-dinitrobenzene (1 mM) as substrates.

Results

Glutathione S-transferase activity in erythrocyte haemolysates

Enzyme activity was studied in haemolysates prepared from two adults, three newborns and two foetuses. The apparent mean specific activity was 0.023 ± 0.004 (S.E.M.) A_{340} unit/min per mg of protein. However, since addition of haemolysate (10 μl; about 150 μg of protein) to the enzyme-assay mixture, in the absence of either or both of the substrates, resulted in a similar decrease in A_{340}, this apparent activity in crude haemolysate was therefore not further studied.

Apparent enzyme activity in plasma from the subjects was variable (0–0.11 A_{340} unit/min per mg of protein) and appeared to depend on the amount of erythrocyte haemolysis.

Glutathione S-transferase activity during intra-uterine life

Enzyme activity measured in the fractions from CM-Sephadex was present in erythrocytes after 12 weeks gestation and was found throughout the 40-week gestational period. The specific activity of the enzyme in foetal erythrocytes was similar to that in adult erythrocytes (Fig. 1).

Comparison of the glutathione S-transferases from erythrocytes of adults and foetuses

(*a*) *Effect of enzyme concentration.* Various amounts (20–150 μl; about 1.5 mg of protein/ml) of the combined enzyme-containing fractions from CM-Sephadex were added to the enzyme-assay mixture; for all preparations, the reaction rate increased in direct proportion to the amount of enzyme added.

(*b*) *Effect of temperature.* The temperature of the enzyme-assay solution was varied, at pH 6.5, between 11°C and 60°C in increments of approx. 5°C; for both the foetal and adult enzyme, maximum activity occurred at 45°C. The data gave linear Arrhenius plots between 11°C and 36°C; the energies of activation of the reaction are shown in

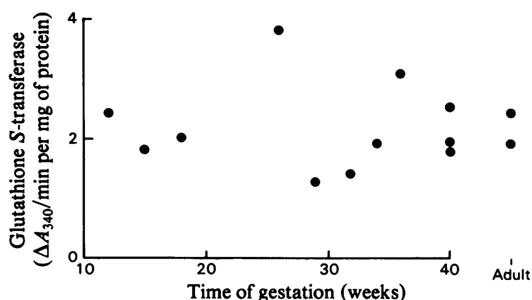

Fig. 1. *Erythrocyte glutathione S-transferase activity during intra-uterine life*

Haemolysate, prepared from foetal (12–40 weeks gestation) and adult erythrocytes, was eluted from CM-Sephadex and the column fractions containing glutathione *S*-transferase activity were combined. The activity of these combined fractions was expressed as A_{340} units/min per mg of protein.

Table 1. The energy of activation of the non-enzymic reaction was 63.0 ± 7.8 kJ · mol^{-1} (S.E.M.; $n = 4$).

(*c*) *Effect of pH.* The pH of the enzyme-assay mixture was altered between 6.0 and 8.0; both foetal and adult enzyme preparations demonstrated maximum reaction rates at pH 6.90. Since the rate of the non-enzymic reaction was substantial above pH 6.8, assays were routinely performed at pH 6.5.

(*d*) *Effect of varying substrate concentrations.* Apparent Michaelis constants (K_m) were derived by varying the concentration of GSH (0.3–3.0mM) at a fixed concentration of 1-chloro-2,4-dinitrobenzene (1mM) and varying the concentration of 1-chloro-2,4-dinitrobenzene (0.2–1.6mM) at a fixed concentration of GSH (3mM). The apparent K_m values for the adult and foetal enzymes were similar (Table 1).

(*e*) *Effects of substrate on enzyme stability.* Portions (50μl) of the enzyme-containing solution were incubated at 25°C for various time periods up to 25min, either alone or in the presence of GSH (1mM) or 1-chloro-2,4-dinitrobenzene (1mM). At 2min intervals, the missing substrate(s) was added and, after mixing, enzyme activity was measured. In each case, enzyme activity fell during the 25min incubation period; the rate of fall was similar in the foetal, neonatal and adult enzyme preparations. The presence of GSH stabilized enzyme activity; after 25min incubation, 88–95% of initial activity remained. In the absence of either substrate 55–68% of initial activity remained, whereas in the presence of 1-chloro-2,4-dinitrobenzene only 36–44% of initial activity remained. For all the enzyme preparations studied, a plot of log activity versus time gave straight lines; this is consistent with the presence of only one enzyme species.

Determination of molecular weight of the glutathione S-transferase in haemolysate

Enzyme activity in haemolysate from foetal, neonatal and adult blood was eluted from Sephadex G-75 as a single symmetrical peak of enzyme activity. The molecular weights calculated from the elution volumes of the protein standards are shown in Table 1.

Discontinuous sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

To study the subunit composition of the erythrocyte enzyme, the mobilities of protein bands in the partially purified preparations of erythrocyte glutathione *S*-transferase were compared with those of the Ya, Yb and Yc monomer bands from rat liver (Hayes *et al.*, 1979). The erythrocyte preparations from foetal, neonatal and adult blood showed the presence of protein bands with the same mobilities as the Ya, Yb and Yc monomers; densitometry of the gels (Bass *et al.*, 1977; Hayes *et al.*, 1979) showed that, in the erythrocyte enzyme preparations, the Yc band contained 85%, the Yb band 10% and the Ya band 5% of the YaYbYc protein. This suggests that the erythrocyte glutathione *S*-transferase comprises two identical monomers, which have the same mol.wt. (25000) as the Yc monomers from rat liver cytosol.

DEAE-Sephadex chromatography

In the foetal, neonatal and adult enzyme preparations, glutathione *S*-transferase activity was initially retained by the ion-exchanger; enzyme activity was eluted as a single peak only after application of the NaCl gradient. Enzymes from adult, foetal and neonatal erythrocytes were eluted at 175mM-, 165mM- and 160mM-Na^+ respectively.

Glutathione S-transferase activity in the haemolysate 100000g pellet

Suspensions of the 100000 *g* pellet, prepared from foetal, neonatal and adult erythrocytes, did not contain detectable glutathione *S*-transferase activity. Unlike glutathione *S*-transferase activity in rat liver microsomal fraction (Morgenstern *et al.*, 1979),

Table 1. *Comparison of the catalytic properties of glutathione S-transferase from foetal and adult erythrocytes*
Erythrocytes from adults (2), newborns (3) and foetuses (2) were lysed and the haemolysate, prepared as described in the Materials and Methods section, was eluted from CM-Sephadex. The fractions containing glutathione *S*-transferase activity were combined and the catalytic properties of the enzyme were examined. The results shown are mean values.

	Foetal (15–18 weeks gestation)	Foetal (36–40 weeks gestation)	Adult
K_m for GSH (mM)	0.10	0.10	0.14
K_m for 1-chloro-2,4-dinitrobenzene (mM)	1.36	1.00	0.75
pH optimum	6.90	6.90	6.90
Temp. optimum (°C)	45	45	45
Activation energy (kJ·mol^{-1})	37.0	42.7	44.7
Mol.wt. (gel chromatography)	48000	49000	47000
Mol.wt. (electrophoresis)	50000	50000	50000

the putative enzyme activity in the erythrocyte 100000*g* pellet was not stimulated by preincubation with idoacetamide.

Discussion

The experiments described show that glutathione *S*-transferase activity is present in foetal erythrocytes after 12 weeks gestation and that the specific activity of the enzyme appears to be constant throughout intra-uterine life and similar to the specific activity found in adult erythrocytes. Marcus *et al.* (1978) found only one enzyme with glutathione *S*-transferase activity in erythrocytes from adults; the behaviour, on ion-exchangers, of the activity from both foetal and adult erythrocytes was similar and also indicates that only one enzyme with glutathione *S*-transferase activity is present in erythrocytes. In this respect, the erythrocyte differs from the liver; there appear to be at least five glutathione *S*-transferases in adult human liver, although it is likely that they result from deamidation *in vivo* of a single parent enzyme rather than by transcription of different genes (Habig *et al.*, 1976).

A comparison of the catalytic properties of the foetal and adult enzymes suggests that throughout intra-uterine life there exists a single form of the enzyme; thus the pH and temperature optima, K_m values and behaviour on ion-exchange columns and polyacrylamide gels, as well as enzyme stabilities, were similar for foetal, neonatal and adult enzymes. In this respect, the erythrocyte glutathione *S*-transferase is similar to alkaline phosphatase from liver, bone and kidney; these enzymes appear to exist in only one form during gestation (Mulivor *et al.*, 1978). Glutathione *S*-transferase differs from enzymes such as hepatic thymidine kinase and intestinal alkaline phosphatase; during early gestation both these enzymes exist as foetal forms, but after about 20 weeks and 30 weeks gestation respectively, adult forms of the enzymes are synthesized and the foetal forms disappear (Taylor *et al.*, 1972; Bernard *et al.*, 1977; Mulivor *et al.*, 1978).

Glutathione *S*-transferase activity has been detected in microsomal preparations from rat liver (Morgenstern *et al.*, 1979), and activity may also be associated with hepatocyte plasma membranes (Jakoby, 1978). Microsomal glutathione *S*-transferase activity in liver, unlike the cystosolic activity, is stimulated by thiol reagents such as iodoacetamide (Morgenstern *et al.*, 1979). Plasma membranes from foetal and adult erythrocytes do not appear to contain glutathione *S*-transferase activity.

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