whereas both cholesterol and deoxycorticosterone form a type I spectrum. It is unlikely that chlorpromazine inhibits steroidogenesis by inhibiting P-450 availability.

In summary, chlorpromazine treatment was found to decrease adrenal cytosol isocitrate dehydrogenase and malic enzyme activity. Chlorpromazine also had a direct action *in vitro* to inhibit the activity of these enzymes. Desipramine treatment had no effect on the soluble enzymes studied and the concentration needed to inhibit succinate dehydrogenase in the adrenal was similar to that reported for the liver and brain mitochondria enzyme. Chlorpromazine treatment did not decrease P-450 content. It is not known whether the influence of chlorpromazine and desipramine on adrenal enzymes is related to their inhibitory effects on steroid production.

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Glutathione-*S*-transferase in human fetal liver*

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Part of the carcinogenic and mutagenic properties of polycyclic aromatic hydrocarbons have been ascribed to their epoxide metabolites [1–3]. Arene oxides have also been implicated as causative agents in tissue necrosis [4]. Little is known, however about the effects of such epoxides on the human fetus.

In view of the fact that the human fetal liver contains the monooxygenase enzyme system [5] and catalyzes the formation of epoxides [6–8] we have investigated the further metabolism of epoxides in preparations of human fetal liver. Apart from being rearranged in water to form phenols, arene oxides may be enzymatically converted to dihydrodiols by a microsomal epoxide hydratase (EC 3.3.2.3) [9] or to glutathione conjugates by glutathione-*S*-transferase (EC 2.5.1.18) [10] which resides in the soluble fraction of the hepatocytes [11, 12].

Multiple basic [13] and recently a neutral [14, 15] form of the glutathione-*S*-transferase were characterized in the human liver. The enzyme has also been described in erythrocytes [16] and placenta [17]. Although the ontogenic post-natal development of hepatic glutathione-*S*-transferase has been studied in rats [18, 19] there is virtually no information on the development of this enzyme in man apart from one preliminary report on the presence of glutathione-*S*-naphthalene-1,2-oxide transferase in a single human fetal liver specimen [20].

This report describes the results from an investigation of glutathione-*S*-transferase in several different human fetal liver preparations.

Liver specimens were obtained from human fetuses at legal abortion on socio-medical indications by Cesarean section or prostaglandin or ethacridine (Rivanol®) induction. The gestational ages of the fetuses varied between 10 and 27 weeks. One third of the mothers were regular smokers and one (no. 12) received β_2-receptor stimulating agents because of asthma.

Specimens of the livers were excised within 45 min of the death of the fetus and frozen at minus 80° if not used at once. After homogenization of the liver piece in 0.25 M sucrose (1:3, w/v) and centrifugation at 9000 *g* for 10 min the 9000 *g* supernatant was centrifuged for 60 min at 105,000 *g* and the supernatant used for the incubations. All steps were carried out at a temperature of 0–4°.

The enzyme assay was essentially the same as described previously [21] with minor modifications. In brief, incubation mixtures of 100 μl contained 0.1 M sodium pyrophosphate buffer, pH 8.5, cytosolic protein at final concentrations between 2 and 6 mg per ml, and 5 μl of acetonitrile containing various amounts of styrene-7, 8-oxide (SO†, to give a final concentration of 6 mM or, when varied, between 0.5 and 12 mM) and about 100,000 cpm of [7-^{3}H]styrene oxide (Radiochemical Centre, Amersham, U.K., sp. act. 99.6 mCi per mmole). The reaction was started by adding 20 μl distilled water containing various

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† Abbreviations used: SO = styrene-7,8-oxide; K_m = Michaelis–Menten constant; V = velocity of the enzyme reaction.

amounts of glutathione (GSH, to give a final concentration of 10 mM, or when varied, between 0.05 and 25 mM) and incubations were carried out for 4 min at 23° in a shaking water bath. Controls with boiled enzyme fractions were always run in parallell for correction for spontaneous non-enzymatic conjugation. In the standard incubations the SO concentration was kept as low as possible to achieve the highest specific activity possible with respect to SO.

The reaction was stopped by addition of 20 μl of 1.5 M acetic acid in order to reduce the pH and block the enzymatic and spontaneous conjugation of SO with GSH. One ml chloroform was added to the sample and the tubes were shaken for 1 min to extract unreacted SO. Forty μl of incubate (1/3 of the sample) were applied to the loading zone of 19-channel LK5DF silica gel plates (Whatman, Maidstone, U.K.). The loading zone of the thin layer chromatography plates were pretreated with a solution of 30% (v/v) cold styrene oxide and 10% (w/v) styrene glycol in methanol to minimize the adsorption of radioactive SO and glycol [21]. The plates were developed with a 8:2 (v/v) mixture of chloroform and ethyl acetate. The loading zone containing the SO-GSH conjugate was then scraped off and counted in a liquid scintillation spectrometer system after the addition of 2 ml methanol to solubilize the conjugate and 10 ml Instagel®.

[^{3}H]-SO was purchased from the Radiochemical Centre, Amersham, U.K., the GSH from Sigma Chemical Co., London, U.K., and unlabeled SO from Aldrich Chemical Co., Gillingham, U.K. All other chemicals were of the highest purity commercially available.

Calculations. The enzyme kinetic data were interpreted using usual velocity substrate concentration curves, Lineweaver–Burk plots and Hofstee plots [22]. Linear regression analysis was made with a table calculator. Differences between means were tested with Student's *t*-test.

Since a concentration dependency in K_m and V_{max} for the GSH conjugation was observed when the GSH concentration was varied while keeping the SO concentration constant (biphasic curves in a Hofstee plot), the enzyme kinetic constants were calculated according to the following equation:

$$V_{tot} = \frac{V_{max1} \cdot S}{K_{m1} + S} + \frac{V_{max2} \cdot S}{K_{m2} + S}$$

This equation was fitted to the actual values for V_{tot} and S and the calculation of the constants was achieved by nonlinear regression analysis using the BMDP3R program (Health Sciences Computing Facility, University of California). The coefficients of variation for the asymptotic standard deviation were calculated for the K_{m1}, K_{m2}, V_{max1} and V_{max2}. The medians (and ranges) were 27 (24–31), 47 (27–76), 6.2 (5.4–6.5) and 2.0 (1.7—4.0), respectively.

Because of the pH-dependent spontaneous GSH-conjugation of SO, the reaction was run at various pHs in order to find the optimum pH, i.e. the pH at which the difference between the total (spontaneous plus enzymatic) and the spontaneous conjugation is maximal. This maximum was found at pH 8.6 with decreasing net rates both at lower and higher pH.

The enzymatic reaction was found to proceed linearly up to 4 min incubation time and at cytosol protein concentrations below 6 mg/ml. Incubation at 23° yielded higher net rates of conjugation than at 30° and 37°.

The rate of the GSH conjugation of SO was tested at various concentrations of each of the two substrates of the transferase, keeping the other substrate at a constant concentration. Different patterns of relation between the rate and concentration of each of the substrates were found. When the GSH concentration was kept constant at 10 mM and the SO concentration was varied between 0.5 and 12 mM typical Michaelis–Menten kinetics were observed when data were analyzed by Lineweaver–Burk [Fig. 1(a)] and Hofstee plots. The values for the apparent K_m and the V_{max} are given in Table 1 for three representative fetal liver specimens.

In contrast, when the SO concentration was kept constant at 6 mM and the GSH concentration was varied non-Michaelian kinetics were observed. This is demonstrated in three different fetal liver enzyme preparations in Fig. 1(b). The Hofstee plots clearly show a low-affinity and a high-affinity phase which seems to be consistent for all three liver specimens. The K_m and V_{max} was calculated in each case and the values are given in Table 1.

The activity of the glutathione-*S*-transferase was investigated in 17 different human fetal livers (Fig. 2). The average activity was 3.49 (± 0.31, S.E.) nmoles conjugate formed per mg protein per minute. When the values were divided into two groups based on the type of abortion (Cesarean section or induced by Rivanol® or prostaglandin) no significant difference was observed ($P > 0.2$). Nor was there any relation between on one hand the activity and on the other the gestational age or smoking habits. Only one mother (no.12) was treated with different drugs and the activity in the liver specimen was below the average.

Particularly two aspects of our findings deserve to be highlighted: (a) the presence and the possible biological significance of the glutathione-*S*-transferase in the human fetus and (b) the nature of this enzyme with respect to the two-phase kinetics with GSH.

Previous work by different research groups [7, 20] including our own [8, 23, 25] has demonstrated that the human fetal liver is capable of catalyzing the *formation* of epoxides as well as the further *metabolism* of this group of intermediary metabolites. Hydration by microsomal hydratase has been demonstrated with naphthalene-1,2-epoxide [20] and styrene oxide [24, 25] as substrates.

Our findings that the glutathione-*S*-transferase catalyzed conjugation of SO with GSH was operative in all investigated fetal livers indicates that this enzyme develops early in human gestation. The mean rate of SO-conjugation was 3.5 nmoles conjugate formed per mg protein per minute. This is only 1/8 of the activity in human adult liver biopsy samples [21]. The human fetal hepatic SO hydratase activity are in the same range, 5 nmoles per mg protein per min [24, 25]. This compares favourably to a corresponding value of 4.7 in human adult liver [26] although higher values were also reported [27, 28]. *In vitro* activities have, however, doubtful relevance for the *in vivo* situation since substrate levels at the enzyme site, cofactor concentration etc. may differ. It has been reported that the relative importance of the two SO detoxifying pathways may shift during development [29] but the *in vivo* contribution of these pathways to the overall elimination of SO must be studied with another approach, e.g. with isolated perfused organs [30].

The ontogenic development of glutathione-*S*-transferase is difficult to study in man over longer time periods. In animals, a postnatal surge of the glutathione-*S*-transferase activity was reported in the rat liver [18], guinea-pig and rabbit liver [29, 31], in rat serum [32] and in rat ovaries [11]. In the adrenals however, it seemed to be relatively constant at a high level during the postnatal period in the rat [11].

Several exogenous compounds induce the hepatic activity of glutathione-*S*-transferase, e.g. phenobarbital [29, 31], pregnenolone-16α-carbonitrile and TCDD [29]. In our patients no correlation between the fetal activities and the maternal smoking habits was found.

The finding of a high and a low affinity phase of the glutathione-*S*-transferase activity with respect to GSH has great interest from an enzymologic point of view. One explanation may be that the enzyme does not obey Michaelis–Menten kinetics because of random substrate binding to the transferase similar to what has been demonstrated with a glutathione-*S*-transferase from rat [33]. Another explanation, although less plausible may be that

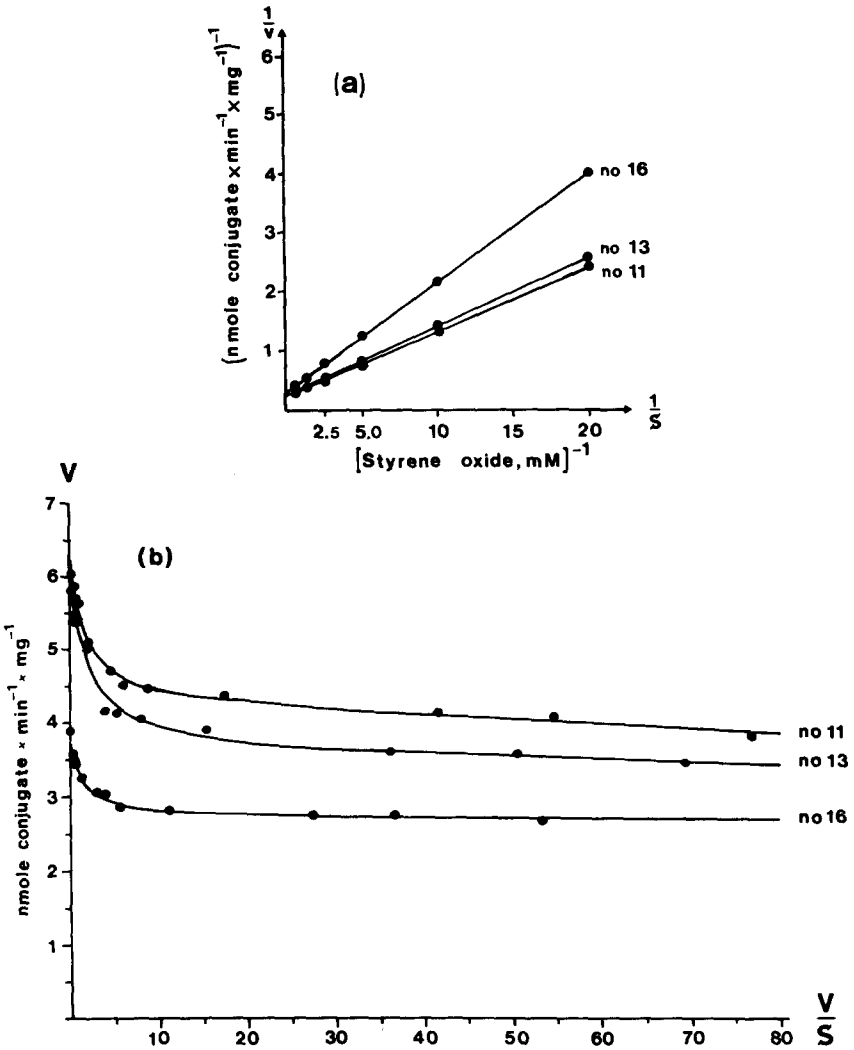

Fig. 1. (a) Glutathione conjugation of styrene oxide at various concentrations of styrene oxide. Lineweaver–Burk transformation of the data. The incubate concentration of styrene oxide ranged from 0.5 to 12 mM, while the glutathione concentration was 10 mM. Final cytosol protein concentration was 5.2, 6.0, 5.7 mg/ml for livers no. 11, 13 and 16, respectively. The reactions were carried out at 23° for 4 min. (b) Non-Michaelian kinetics of the human fetal liver glutathione-*S*-transferase catalyzed glutathione conjugation of styrene oxide at various concentrations of glutathione. Eadie–Hofstee transformation of the data. The incubate concentrations of glutathione ranged from 0.05 to 25 mM, while the concentration of styrene oxide was 6 mM. Final cytosol protein concentrations were 5.2, 6.0 and 5.7 mg/ml in livers no. 11, 13 and 16, respectively. The reactions were carried out at 23° for 4 min.

Table 1. Kinetic parameters of the human fetal hepatic glutathione-*S*-transferase obtained using varying concentrations of glutathione or styrene oxide keeping the concentration of the other substrate constant (6 mM styrene oxide or 10 mM glutathione)

Varying concentration of glutathione	Liver no. 11	Liver no. 13	Liver no. 16
K_{m1} (mM)	3.60	2.45	4.26
K_{m2} (mM)	0.0054	0.0035	0.0027
V_{max1} (nmoles min^{-1} mg^{-1})	2.11	2.37	1.20
V_{max2} (nmoles min^{-1} mg^{-1})	4.29	3.67	2.81
Varying concentration of styrene oxide	**Liver no. 11**	**Liver no. 13**	**Liver no. 16**
K_m (mM)	3.71	4.31	4.56
V_{max} (nmoles min^{-1} mg^{-1})	7.39	7.18	5.04

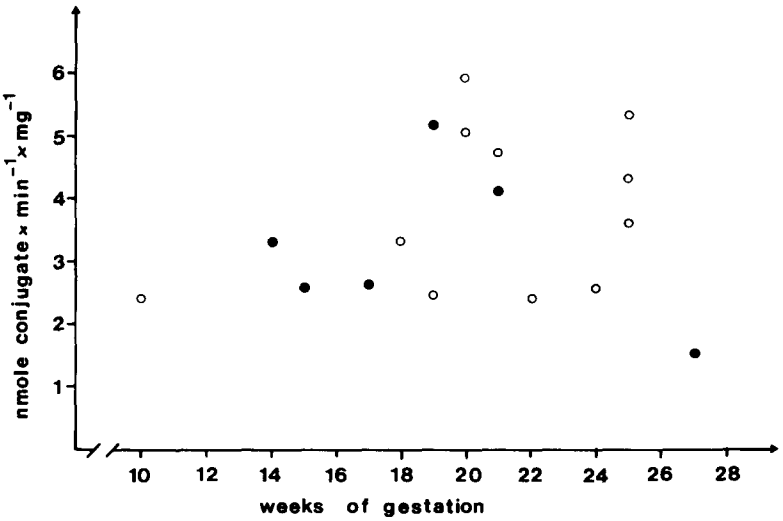

Fig. 2. Glutathione-*S*-transferase activity in 17 human fetal liver cytosols. The tissue specimens were obtained either after Cesarean section (O) or abortion induced by prostaglandins or ethacridine (Rivanol®). Incubate concentrations of styrene oxide and glutathione were 6.0 and 10 mM, respectively. The incubations were carried out at 23° for 4 min.

the enzyme possesses two binding sites for GSH with different affinities. Alternatively, there may be two enzymes, each with different affinity for glutathione but with identical affinity for SO. The differentiation between these possibilities is difficult. Recent preliminary findings (together with B. Mannervik, C. Guthenberg, M. Warholm) indicate that the biphasic kinetics reside in one of two transferase forms.

It is not known whether the fetal transferase acting on SO is related to or identical with any of the glutathione-*S*-transferase species described in human adult liver [13–15]. The possibility certainly exists that the function of one transferase may be taken over quantitatively by another at a later developmental stage since, at least in the rat, the different transferases have partly overlapping substrate specificities [12]. Such a mechanism may partly explain the physiological neonatal hyperbilirubinemia and the low neonatal clearance of bromosulfophthalein [34] in comparison with adults. The maturation of these functions may of course also be due to an increase of the enzyme levels with increasing age.

In summary our data show that one of the major metabolic pathways of epoxides, catalyzed by the glutathione-*S*-transferase, is operative already in mid-gestation in the human fetal liver. This enzyme follows Michaelis–Menten kinetics with varying concentrations of SO whereas non-Michaelian kinetics were observed with varying GSH concentrations. The early development of the enzyme has feto-toxicological/teratological interest in view of the apparent ubiquity of epoxide substrates in our environment.

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The effect of loperamide on the ion fluxes across the isolated rabbit colon

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Loperamide is a phenyl-piperidine derivative, structurally related to haloperidol, which is used to control diarrhoea.

Although loperamide is effective in inhibiting intestinal motility [1] it is also able to reverse diarrhoeas of a secretory origin as for example those due to prostaglandin E_2 [2] and to cholera toxin [3]. These agents act by increasing the activity of adenylate cyclase within the mucosal cells so raising cellular cAMP levels [4]. In the ileum the increased permeability of the apical membrane to Cl^-, which follows the rise in intracellular cAMP, effectively shorts out the high chloride resistance of the tight junctions and allows NaCl to leak out of the lateral intercellular spaces. This movement of solute is followed by water and a secretion of fluid into the lumen results [5].

Secretion may be reversed by several groups of drugs including δ-opiates [6], β-blockers [7], phenothiazines [8] and glucocorticoids [9] but they do not share the same mechanism of action. For example phenothiazines block the above process of chloride secretion by binding to and inactivating the complex between Ca^{2+} and the calcium dependent regulator protein, calmodulin [10] whilst the glucocorticoids increase the uptake of sodium by the tissue so that it is absorbed faster than it is secreted [9].

In an attempt to elucidate the mechanism by which loperamide abolishes secretion we have measured the unidirectional fluxes of sodium and chloride across the *in vitro* rabbit colon, in the presence of loperamide, before and after increasing tissue cAMP levels with theophylline.

The experiments were performed on lengths of descending colon removed from male NZW rabbits of 2–3 kg which had been killed by cervical dislocation and bleeding. The pellets were washed out and the mesenteric fat cleared from the serosal surface. A cut was made down one side of the colon which was then spread out, mucosa upwards on a sheet of perspex. The mucosa, submucosa and lamina propria were stripped from the underlying muscle using a pair of microscope slides. The stripped tissue was carefully mounted across the ports of a block of six flux chambers and the two halves of the block clamped together. The chambers were each filled with 8 ml of Kreb's bicarbonate Ringer solution and gassed with 5% CO_2 in O_2. The chambers were maintained at 38° by an integral water jacket and after mounting the preparation was left for 30 min to allow it to come to equilibrium before flux measurements were made.

The potential difference across the tissue was measured with calomel electrodes from saline–agar bridges placed close to the tissue and during fluxing the potential was clamped to zero by means of an automatic short circuit current apparatus. No correction was made for the resistance of the solution between the tips of the bridges as this did not exceed 3 per cent of the total resistance.

Fluxes were determined using ^{24}Na and ^{36}Cl as labels. These were added sometimes separately, sometimes together to one of the chambers. Mucosal to serosal (m–s) and serosal to mucosal (s–m) fluxes were measured in the same experiment. After two control periods of 15 min either theophylline (final concentration 1.5 mM) or loperamide (final concentration 2×10^{-5} M) was added to the serosal chamber and after two further periods of 15 min the other drug was added. Potential recordings showed that both drugs produced an effect within 30 sec of administration, so flux rates from the first and second periods were combined in the results. The mean rate for each unidirectional flux was calculated and the significance of the changes determined by a *t*-test between the means.

Theophylline was obtained from Sigma Chemical Co. (London, U.K.) and loperamide was the gift of Janssen Pharmaceuticals. ^{24}Na was obtained as the bicarbonate from the University of London Reactor and ^{36}Cl from the Radiochemical Centre (Amersham, U.K.).

It may be seen from Table 1 that under short circuit conditions the rabbit colon shows a net absorption of Na^+ and Cl^-. The unidirectional fluxes of Cl^- are higher than those of Na^+, in particular J^{Na} s–m, the low value of which reflects the high resistance of the leakage pathway in this tissue.

The effect of 1.5 mM theophylline on these fluxes is shown in part A of the table. There is an increase in J^{Na}m–s but the net movement of Na^+ is not significantly different from control because of the increase in J^{Na}s–m and this implies that there is now a leakage of Na^+ across the tight junctions.

Unidirectional Cl^- fluxes have also increased but J^{Cl}s–m