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Differential Transplacental Binding of Diazepam: Causes and Implications

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Summary. Diazepam plasma binding was determined in 17 matched pairs of maternal and foetal plasma, collected at delivery. Diazepam % free was higher ($p < 0.001$) in maternal (mean 3.24%) than in either umbilical venous (mean 1.50%) or umbilical arterial (mean 1.24%) plasma. The data from in vitro dialysis studies were consistent with the reported higher diazepam concentrations in infants than in mothers at delivery. Plasma nonesterified fatty acids (NEFA) concentrations were higher ($p < 0.001$) in maternal ($\bar{x} = 643$ μM) than in matched umbilical venous plasma ($\bar{x} = 211$ μM) and there was a significant correlation ($p < 0.01$) between diazepam % free and corresponding plasma NEFA concentration for pooled data ($r = 0.871$, $n = 34$). Multiple and partial regression analysis indicates that transplacental differences in albumin, bilirubin and total protein concentrations made a minimal contribution to diazepam binding differences between mother and foetus and that approximately 76% of the variability in diazepam % free was accounted for by plasma NEFA concentration. The binding of diazepam to human serum albumin (HSA) was markedly perturbed by the presence of NEFA but not by bilirubin and there was no apparent cooperativity between bilirubin and NEFA on diazepam-HSA binding. Moreover, our findings provide further evidence that substantial differences in binding affinities exist between foetal and maternal plasma albumins.

Key words: diazepam, fatty acid binding; matched maternal and foetal plasma, plasma NEFA concentration, plasma bilirubin, plasma albumins

Diazepam is used extensively in the management of maternal eclampsia and severe pre-eclampsia but crosses the placenta [1–6] to accumulate in the foetus; after birth, it is only slowly metabolized and excreted [7]. The clinical status of the newborn, following high maternal doses of diazepam, is reported to be poor [2, 7].

Total diazepam plasma levels in the foetus, at delivery, have generally been reported to be higher than in the mother [1–3, 6]. These observations have been interpreted variously in terms of the kinetics of drug transfer between mother and foetus [3], with the foetus exhibiting 'deep compartment' characteristics [8], or in terms of differences in plasma protein binding between mother and foetus [6, 9]. Studies, in vitro [10] and in vivo [1, 4], indicate that the placental passage of diazepam is rapid, and it is improbable, therefore, that the foetus acts as a 'deep compartment'. However, there is apparent conflict among reported estimates of diazepam binding. Both higher [5] and lower [9, 11] extents of binding have been reported in maternal, with respect to foetal, plasma.

The present investigation examined plasma binding and its influence on the observed foetal: maternal diazepam concentration ratio at delivery. Factors contributing to the differences between maternal and foetal plasma binding were investigated. An equilibrium dialysis technique, free from in vitro alterations in plasma pH and NEFA concentrations [16], was employed.

Subjects, Materials, Methods

Subjects

Subjects were 17 pregnant women, and their newborn infants, at delivery. All mothers, aged 19 to 39 years, were of normal weight (range 58 to 76 kg)

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Table 1. Diazepam binding in maternal and foetal plasma

Patient	Maternal % free	Umbilical venous % free	Umbilical arterial % free	Calculated[a] F/M ratio	Oberved[a] F/M ratio
1	3.55	1.49	–	2.38	–
2	2.92	1.44	–	2.04	2.13
3	2.81	1.28	1.23	2.17	–
4	3.11	1.89	1.38	1.64	1.69
5	4.80	2.30	1.06	2.08	2.08
6	2.03	1.17	0.91	1.72	1.69
7	2.99	1.73	0.90	1.72	–
8	4.60	1.35	1.23	3.45	3.33
9	4.21	1.70	1.65	2.50	–
10	2.37	1.32	–	1.78	1.75
11	2.52	1.47	1.42	1.72	–
12	3.21	1.69	1.60	1.89	1.85
13	2.55	1.43	1.31	1.79	–
14	1.18	0.94	–	1.25	1.27
15	3.08	1.27	1.16	2.44	2.56
16	3.13	1.27	1.07	2.50	2.56
17	6.10	1.80	–	3.33	–
mean ± SD	3.24 ± 1.15	1.50 ± 0.32	1.24 ± 0.24	2.14 ± 0.58	
		$p < 0.001$	$p < 0.01$		
			$p < 0.001$		

[a] F/M ratio = umbilical venous/maternal venous diazepam concentration ratio

and in good health, with the exception of 1 asthmatic and 1 subject suffering from pre-eclamptic toxaemia. Subjects were primiparous (9) and multiparous (8) and infants were of mean gestational age 38.9 weeks. Eleven women delivered by elective caesarean section; 9 receiving bupivacaine or etidocaine epidurally and 2 receiving general anaesthesia by thiopentone and nitrous oxide. Six women delivered vaginally; 4 receiving bupivacaine epidurally for pain relief. Other drugs administered were as follows (number of subjects indicated in parentheses): apresoline (1); betamethasone (1); chlormethiazole (1); diazepam (5); hyoscine (1); metoclopramide (1); synthetic oxytocin (2); and salbutamol (1).

Blood Collection

With informed consent, blood samples (10 ml) were drawn from a maternal peripheral vein at delivery, and from the umbilical vein and artery of the doubly-clamped cord after delivery of the placenta. The samples were collected by venipuncture in disposable plastic syringes (Monoject, Sherwood Medical Ind., Florida) and transferred to heparinized plastic tubes (Disposable Products, Australia) for immediate centrifugation at 4 °C. Plasma was analysed immediately or stored frozen at −22 °C for a maximum of 7 days. Concentrations in maternal and foetal plasma of total protein and albumin (Rapid Stat Kit, Pierce) and total bilirubin (A-Gent Bilirubin Test, Abbott) were measured. Plasma nonesterified fatty acids concentrations were quantified by gas chromatography [15].

Estimation of Plasma Binding of Diazepam

The plasma binding of ^{14}C diazepam (specific activity 186 μCi/mg; Roche Products, Basle) was estimated by equilibrium dialysis in 1 ml PTFE half dialysis cells (Spectrum Medical Industries, Inc., California) separated by a Spectrapor 2 membrane (Spectrum Medical Industries Inc., California). Plasma (1 ml) was spiked with diazepam 400 ng/ml and dialysed to equilibrium against an equal volume of Sörensens phosphate buffer (0. 067 M, pH 7.4) at 37 °C for 2 h. Aliquots of plasma and buffer were mixed with 10 ml Insta-gel (Packard Instrument Co.) and counted in a Hewlett-Packard model 3255 Scintillation Spectrometer, using external standard ratio quench correction. Diazepam free fraction was obtained by calculating the ratio of the concentration of diazepam in the buffer compartment to the concentration of diazepam in the plasma compartment and expressed as a percentage. Radiochemical purity (99.6%) was established for total (by TLC) and for free (by TLC and HPLC) diazepam. The plasma binding methodology was reproducible (C.V. = 3.8%, $n = 5$, mean % free 0.82). Prior experiments had established that

Table 2. Concentrations of plasma NEFA, plasma proteins and bilirubin in maternal and umbilical (foetal) venous samples

Patient pair	NEFA [μM]		Total protein [g/l]		Albumin [g/l]		Total bilirubin [μM]	
	maternal	foetal	maternal	foetal	maternal	foetal	maternal	foetal
1	610	154	51	53	31	44	8	34
2	618	217	68	72	42	44	15	31
3	770	203	61	54	33	29	12	17
4	540	113	62	56	40	39	11	26
5	903	253	63	51	37	38	13	35
6	525	185	57	63	32	49	16	48
7	692	198	66	55	37	38	12	30
8	598	188	61	50	34	40	20	34
9	760	258	48	55	34	41	13	36
10	490	205	57	64	33	42	9	27
11	686	220	90	76	45	38	10	24
12	600	225	75	69	39	40	10	19
13	600	359	61	90	35	46	7	37
14	438	112	65	48	36	31	6	13
15	568	370	58	57	34	38	7	30
16	416	170	58	66	31	43	5	21
17	1121	162	70	75	31	41	9	25
mean ± SD	643 ± 174	211 ± 7	63 ± 10	62 ± 16	36 ± 4	40 ± 5	11 ± 4	29 ± 9
	$p < 0.001$		$p > 0.1$		$p < 0.01$		$p < 0.001$	

this methodology was free from interference by in vitro lipolysis and plasma pH changes [16].

Influence of Bilirubin and NEFA on Diazepam Binding to HSA

Bilirubin (B.D.H. Laboratory Chemicals), 0.75 mg/ml solution in 0.1 M NaOH, was incorporated into 3.5% human serum albumin solutions (HSA; Behringwerke Marburg/Lahn) to achieve final bilirubin concentrations of 12 μM and 30 μM. Palmitic and oleic acids (Sigma Chemical Co.) were introduced in equimolar concentrations to a 3.5% HSA-buffer solution, using a Celite addition technique (Celite 545, Johns Manville Inc.) [17], to obtain a final total NEFA concentration of 700 μM; a background NEFA concentration of 150 μM was shown to pre-exist in the HSA solution. Various combinations of high and low NEFA and bilirubin concentrations were thereby achieved for 3.5% HSA solutions. The protein binding of ^{14}C diazepam was estimated in these solutions at 50, 100, 200 and 400 ng/ml. All bilirubin experiments were conducted in the dark. A Celite-treated 3.5% HSA solution, to which appropriate amounts of 0.1 M NaOH had been added, served as a control.

Distribution of Bilirubin and NEFA Between Maternal and Foetal Plasma in Vitro

The plasma concentrations of bilirubin and NEFA were monitored serially for 2 subjects during dialysis of matched maternal plasma (1 ml) against umbilical venous plasma (1 ml) at 37 °C for periods up to 5 h. A separate dialysis cell was sampled at each time point. Bilirubin and NEFA concentrations were similarly monitored during dialysis of a 3.5% HSA solution containing 12 μM bilirubin and 700 μM NEFA (typical of maternal plasma levels) against a 3.5% HSA solution containing 30 μM bilirubin an 150 μM NEFA (typical of foetal plasma levels) at 37 °C for 5 h.

Statistical Analysis

Statistical comparisons between maternal and foetal groups were made using the Mann-Whitney U test, two tailed [18]. Correlation analysis between diazepam binding estimates and biochemical variables were performed by linear, partial and multiple regression procedures [18]. The 0.05 level was regarded as the limit of statistical significance.

Results

The binding of diazepam in matched maternal and foetal plasma is shown in Table 1. Considerable interindividual variability was exhibited in extents of binding in all plasma groups but was larger for maternal (CV = 35%) than for umbilical (foetal) venous (CV = 21%) or umbilical arterial (CV = 19%) plasmas. Diazepam % free was greater in maternal plas-

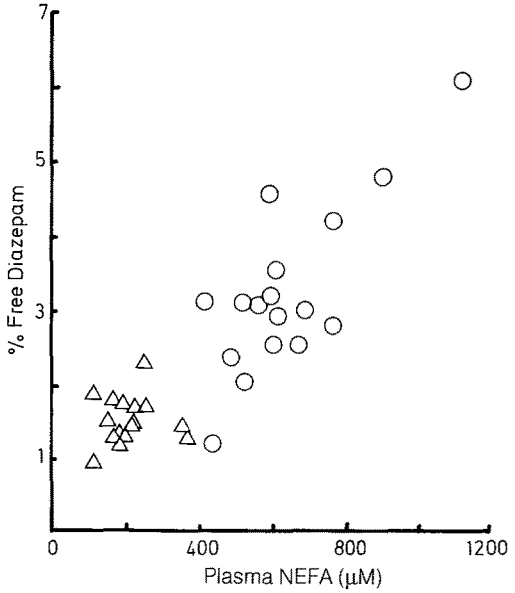

Fig. 1. Relationship between diazepam % free and plasma NEFA concentration in maternal (○) and umbilical venous (△) plasmas. Regression equation is $y = 0.600 + 0.004x$

Table 3. Diazepam % free determined in HSA solutions on addition of bilirubin and NEFA

3.5% HSA solution[a]		% Free			
Bilirubin (μM)	NEFA (μM)	Diazepam concentration [ng/ml]			
		50	100	200	400
–	150	0.37	0.39	0.37	0.39
–	700	2.53	2.69	2.87	2.59
12	150	0.39	0.37	0.39	0.39
30	150	0.40	0.39	0.39	0.38
12	700	2.88	2.71	2.52	2.81
30	700	2.81	2.89	2.69	2.73

[a] 3.5% HSA solution had a pre-existing NEFA concentration of 150 μM

ma than in umbilical venous ($p < 0.001$) plasma. Smaller differences ($p < 0.01$) in diazepam % free were observed between umbilical venous and arterial plasmas.

Preliminary experiments had established that direct equilibration of maternal plasma against umbilical venous plasma (both containing 400 ng/ml ^{14}C diazepam) required a prolonged equilibration time and would thus be subject to interference from in vitro lipolysis [15]. Therefore, using % free data obtained from separate rapid dialyses of maternal and foetal plasmas against buffer, a foetal/maternal diazepam total concentration ratio was calculated (Table 1) assuming equilibrium of unbound concentrations on both sides of the membrane. As an experimental test of this ratio 10 matched maternal and foetal plasma pairs were spiked with diazepam concentrations at the calculated foetal/maternal ratio and dialysed against each other for 2 h at 37 °C. The diazepam concentration ratio in the dialysed foetal and maternal plasmas remained essentially unchanged after 2 h (Table 1) indicating that the true foetal/maternal concentration ratio very closely approximated the calculated value.

Variability in the plasma concentrations of albumin, total protein and potential endogenous displacing agents such as bilirubin and NEFA were examined, by linear, partial and multiple regression analyses, as modulating factors of diazepam % free.

Concentrations of plasma NEFA were higher ($p < 0.001$) in maternal than in umbilical venous (foetal) plasma (Table 2). With pooled maternal and foetal data, a significant linear correlation was demonstrated between diazepam % free and corresponding plasma NEFA concentration ($r = 0.871$, $p < 0.01$, $n = 34$), as is shown in Fig. 1. Plasma albumin concentrations were higher ($p < 0.01$) in foetal than maternal plasma (Table 2). A weak inverse linear correlation between diazepam % free and plasma albumin concentration was apparent ($r = -0.418$, $p < 0.02$, $n = 34$) in the pooled data indicating that some 17% of the variation in diazepam % free could be attributed to variation in plasma albumin level. Plasma total protein concentration was not different ($p > 0.05$) between mothers and their infants (Table 2) and did not correlate ($p > 0.05$) with diazepam % free ($r = 0.007$, $n = 34$). Multiple linear regression of diazepam % free on plasma NEFA and total protein concentration (multiple $R^2 = 0.777$, $n = 34$) and diazepam % free on plasma NEFA and albumin concentration (multiple $R^2 = 0.732$, $n = 34$) gave coefficients of determination which were very similar to that obtained by linear regression of diazepam % free on plasma NEFA concentration.

Plasma bilirubin concentration was higher ($p < 0.001$) in foetal than in maternal plasma (Table 2) and was correlated with diazepam % free ($r = -0.527$, $p < 0.01$, $n = 34$). However, this correlation probably reflects an inverse association ($p < 0.01$) between bilirubin and NEFA concentrations. Partial regression of diazepam % free on plasma bilirubin concentration with plasma NEFA concentration held fixed produced no significant correlation ($r = 0.073$, $p > 0.05$, $n = 34$). Furthermore, no improvement in the correlation of diazepam % free with plasma NEFA concentration ($r = 0.871$) could be demonstrated by multiple regression with the additive variable of plasma bilirubin concentration (multiple $R^2 = 0.759$, $n = 34$).

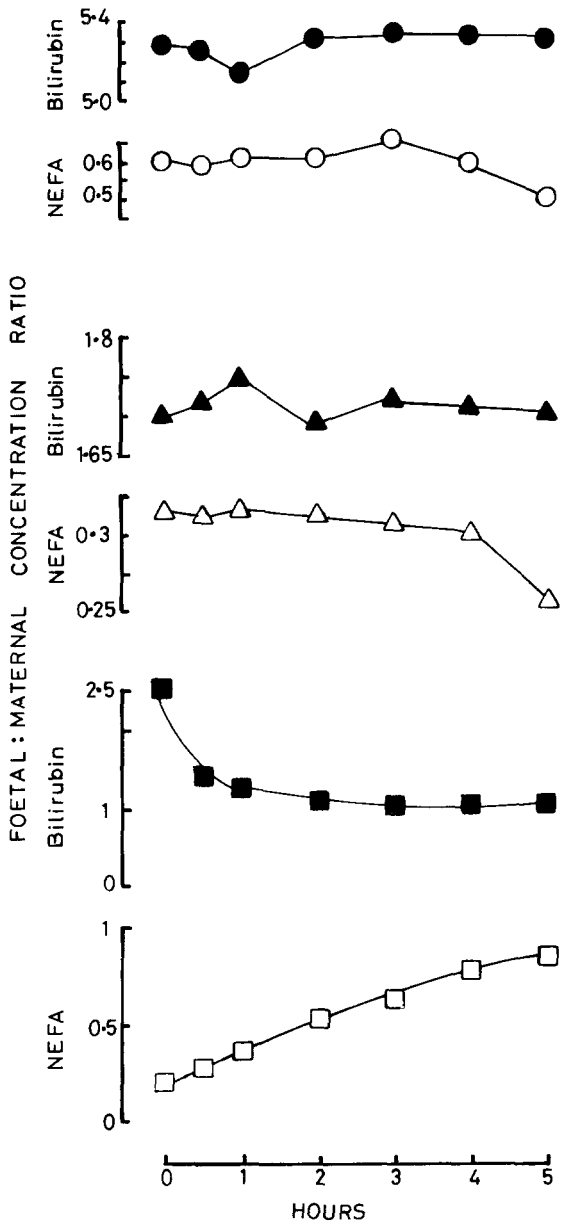

Fig. 2. Variation in foetal: maternal concentration ratios of bilirubin (solid symbols) and NEFA (open symbols) during dialysis. Maternal plasma was dialyzed against matched umbilical venous plasma, for 2 women (circles, triangles). Dialysis of 3.5% HSA solutions, containing bilirubin and NEFA in concentrations typical of maternal and foetal plasmas, served as a control (squares)

Causality between plasma NEFA and/or bilirubin concentration and diazepam % free was examined using 3.5% human serum albumin (HSA) solutions in phosphate buffer. Diazepam % free (mean 0.39%) was shown to be constant over the diazepam concentration range 50 to 400 ng/ml in the presence of 0, 12 and 30 μM bilirubin and 150 μM NEFA (Table 3). A sevenfold increase in diazepam % free occurred with NEFA concentration increase from 150 to 700 μM (Table 3). Diazepam % free (mean 2.73%) was constant in the presence of 0, 12 and 30 μM bilirubin and 700 μM NEFA.

No membrane transfer of bilirubin or NEFA was observed during dialysis of maternal against foetal plasma for 5 h in two subject pairs (Fig. 2). A decrease in foetal: maternal NEFA concentration ratio was observed in both instances between 4 and 5 h dialysis. This probably reflects lipolysis known to occur during prolonged dialysis of plasma from parturients [15]. In contrast, when 3.5% HSA solutions containing NEFA and bilirubin, in concentrations typical of maternal and foetal plasmas, were placed on both sides of the dialysis membrane, both compounds crossed the membrane and equilibrated readily (Fig. 2).

Discussion

Diazepam plasma binding was markedly lower in mothers than in their newborn infants (Table 1). Considerable variability existed in previous reports of diazepam plasma binding in mothers and infants [5, 9, 11]. These in vitro binding data imply that diazepam plasma concentration would be considerably higher in the infant than in the mother at delivery and are in agreement with the majority of transplacental studies which demonstrate a diazepam ratio foetal/maternal at delivery ranging from 1.0 to 3.0 [1–6]. Moreover, when maternal plasma was dialyzed against matched umbilical venous plasma, the diazepam concentration ratio foetal/maternal remained in this range (Table 1).

Furthermore, differences in extent of diazepam binding appear to exist in the foetal circulation. A significantly lower diazepam % free was observed for umbilical arterial with respect to umbilical venous plasma and the former is a more accurate indication of the amount of free drug leaving foetal tissues.

Numerous investigators have considered the factors likely to be responsible for the differential drug plasma binding between mother and foetus, [9, 11, 19, 20]. In the present investigation, plasma total protein, albumin, bilirubin and NEFA concentrations, and the existence of qualitative differences in foetal and maternal plasma albumin, were considered as influential factors in diazepam plasma binding.

A strong linear correlation was observed for pooled data between diazepam % free and plasma NEFA and 75.8% of the variability in diazepam % free was attributable to plasma NEFA concentration variability. On multiple regression, 73.2% and 77.7%

% free was associated with plasma NEFA and albumin, and plasma NEFA and total protein, respectively. Moreover, plasma NEFA levels were markedly ($p < 0.001$) higher in mothers than in infants. These statistical data strongly suggest that NEFA is an important contributing factor in transplacental binding differences of diazepam. The plasma binding of diazepam is known to be perturbed in the presence of NEFA [12, 13] and plasma NEFA concentration has previously been shown to be a determining factor in the maternal binding of diazepam during parturition and the perinatal period [14].

Inverse correlations were observed for diazepam % free with albumin ($r = -0.418$, $p < 0.02$, $n = 34$) and with bilirubin ($r = -0.527$, $p < 0.01$, $n = 34$) but not with total protein ($r = 0.007$, $p > 0.05$, $n = 34$). Partial regression analyses, however, demonstrated no significant correlation ($p > 0.05$) between diazepam % free and albumin with NEFA held fixed ($r = 0.184$, $n = 34$) and between diazepam % free and bilirubin with NEFA held fixed ($r = 0.073$, $n = 34$). These data are inconsistent with the previous speculation that diminished maternal plasma albumin concentration was responsible for reduced diazepam binding at parturition [11] and it is unlikely that the reduced albumin concentration observed in mothers with respect to infants (Table 2) is of any consequence in the differential plasma binding of diazepam. The possibility of a cooperative effect between plasma NEFA and bilirubin on diazepam plasma binding cannot, however, be excluded on the basis of these statistical data.

The binding of diazepam to HSA, its major binding protein [21], was shown to be markedly perturbed in the presence of NEFA but not of bilirubin (Table 3). Nor was there any apparent cooperativity of bilirubin and NEFA on diazepam-HSA binding. A similar lack of effect of diazepam on bilirubin binding has been reported [19].

Data of Tables 1 and 3 establish that the differential plasma NEFA concentration is an important determinant of diazepam plasma binding differences between mother and infant. Therefore plasma NEFA concentration must be a dominant factor governing the postdistributive diazepam plasma concentration ratio between mother and foetus. The contribution of albumin, total protein and bilirubin concentrations appears to be minimal. A lack of correlation of diazepam % free with urea, creatinine, uric acid, cholesterol and triglycerides has been shown previously for plasma from parturients [14]. Even so, the physiological and hormonal changes accompanying parturition are complex and other unidentified factors may contribute to transplacental binding differences of diazepam.

Umbilical and maternal plasmas were collected simultaneously at delivery and were shown to contain substantially different concentrations of both bilirubin and NEFA (Table 2). It can be assumed that these compounds in the matched plasmas were at distributional equilibrium. When the umbilical venous plasma was dialysed against maternal plasma for periods up to 5 h, no shift from the in vivo equilibrium concentrations of bilirubin or NEFA occurrred and the foetal; maternal concentration ratios remained essentially unchanged from 0 to 4 h dialysis (Fig. 2). Bilirubin [22] and NEFA [23] are thought to bind exclusively to albumin, present in maternal and foetal plasmas in approximately equal concentrations (Table 2), and both are capable of crossing the dialysis membrane (Fig. 2). It may be concluded, therefore, that maternal and foetal albumins have different binding affinities for bilirubin, NEFA, and possibly diazepam. This proposal is further substantiated by the ready equilibration of bilirubin and NEFA during dialysis of 3.5% HSA solutions containing concentrations of both species typical of those in maternal and foetal plasmas (Fig. 2).

Plasma albumin in the newborn has been shown to differ from that of the adult by isomerization studies [24] and in amino acid content and composition [25]. Furthermore, reduced [26] and enhanced [19] drug binding capacity of albumins from newborns, relative to adults, has been reported. It is possible, however, that the concentration ratio foetal/maternal of bilirubin and NEFA (Fig. 2) are maintained during dialysis by the presence of a nondialyzable binding inhibitor, but there is no corroborative evidence for such a hypothesis.

A significant correlation ($p < 0.001$) between diazepam % free and plasma NEFA concentration was demonstrated for pooled data (Fig. 1) and for maternal data ($r = 0.778$, $n = 17$) but not for umbilical venous data ($r = 0.063$, $p > 0.05$, $n = 17$). It would appear, therefore, that NEFA concentration is not an important modulating factor in diazepam binding in foetal plasma. The differences displayed between maternal and foetal plasmas in NEFA concentration, and in the correlation between NEFA concentration and diazepam % free, further support the existence of qualitative differences between foetal and maternal plasma albumin.

In conclusion, therefore, marked differences in extent of diazepam plasma binding exist between mothers and their newborn infants. The plasma binding data are consistent with reports of higher diazepam plasma concentration in the infant than in the mother at delivery. In 3.5% HSA solutions, alterations in diazepam % free were produced by changes in NEFA but not of bilirubin concentration. Trans-

placental differences in albumin and total protein concentrations made a minimal contribution to differential diazepam binding between mother and foetus. Moreover, our findings provide further evidence that foetal and maternal plasma albumins differ substantially in their binding affinities.

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