sponse to suboptimal doses of insulin (*9*). Epidermal growth factor will induce receptor aggregation, whereas a competitive antagonist of epidermal growth factor will bind, but not cause, receptor aggregation (*10*). These studies suggest that aggregation of receptors or receptor-ligand complexes into groups of two or more may be essential for biological response.

Transduction of the chemotactic signal may also be dependent on aggregation of cell-bound peptide or peptide-receptor complexes into clusters of two or more. In this way, the intracellular signal would be proportional to the square or some higher power of the concentration of occupied receptors. This would serve to amplify small differences in receptor occupancy across a cell's dimensions when the cell is exposed to a concentration gradient of chemoattractant and may be important for the recognition of gradient directionality.

The role of internalization is also uncertain. Although only the TMR-peptide was seen to be internalized, studies with the ^{125}I-labeled formyl peptide demonstrated the simultaneous loss of cell surface binding. This suggests that the peptide-receptor complex was internalized as a unit or that the surface receptors were inactivated by the initial binding event, perhaps because of masking by secretory or hydrolytic products or a conformational change leading to decreased affinity. The absence of cell surface binding persisted when the washed cells were incubated for 2 hours at 37°C. This process of internalization or inactivation may simply clear occupied receptors from the cell surface so that the cell can respond to changes in the orientation of the chemotactic gradient. Alternatively, the formyl peptides induce many other cell responses including superoxide production, lysosomal enzyme release, enhanced phagocytosis, and cell agglutination (*11*) which may be dependent upon internalization of the peptide or peptide-receptor complex. Receptor internalization or inactivation may explain the phenomenon of neutrophil deactivation, in which cells exposed to saturating concentrations of a chemotactic factor and subsequently washed extensively, are found to be unresponsive to the factor to which they have been exposed previously, but fully responsive to other, chemically dissimilar, chemotactic factors (*12*).

The TMR-peptide will facilitate several types of experiments. Binding to individual cells can be determined, thus the formyl peptide receptor can be assayed on cells that cannot be obtained in a pure form for radioligand studies. More important, occupied receptor distribution can be determined in the presence of a TMR-peptide gradient to test the differential receptor occupancy theory directly (*13*).

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5. Tetramethylrhodamine isothiocyanate (10 m*M*), purified by thin-layer chromatography in a mixture of *n*-butanol, acetic acid, and water (4:1:1 by volume), R_F = 0.65, was reacted with *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys (2.5 m*M*) in dry dimethylformamide, containing 1 percent triethylamine for 18 hours at 22°C. The product (TMR-peptide) was purified by thin-layer chromatography in a mixture of chloroform, methanol, and triethylamine (5:2:1 by volume), R_F = 0.31.
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7. The *N*-formyl-Nle-Leu-Phe-Nle-[^{125}I]Tyr-Lys was prepared by reacting 1 nmole of formyl peptide with 10 nmole of chloramine T and 2 to 3 mCi of carrier-free ^{125}I for 10 minutes at 22°C in 100 μl of 250 m*M* sodium phosphate, *p*H 7.6, and purified on a Bio-Gel P-2 column. The tetramethylrhodamine conjugate was prepared by reacting 150 pmole of *N*-formyl-Nle-Leu-Phe-Nle-[^{125}I]Tyr-Lys (800 Ci/mmole) with 1 μmole of tetramethylrhodamine isothiocyanate in a mixture of 100 m*M* sodium phosphate, *p*H 8.0, and dimethylformamide (1:1 by volume) for 2 days at 22°C. The product, [^{125}I]TMR-peptide, was purified by thin-layer chromatography in a mixture of chloroform, methanol, and triethylamine (5:2:1 by volume), R_F = 0.36. It bound to the neutrophil formyl peptide receptor in a saturable manner with an EC_{50} of 2.0 n*M*.
8. Neutrophils (10^6 cells), which had been exposed to the [^{125}I]TMR-peptide for 3 minutes or 10 minutes at 37°C, were washed three times with 5 ml of phosphate-buffered saline (4°C) and extracted three times into 1.0 ml of a mixture of chloroform and methanol (1:2 by volume) with sonication and gentle warming. The purified [^{125}I]TMR-peptide and the extracted radioactive peptide were analyzed by thin-layer chromatography and autoradiography. They displayed identical R_F values in the following mixtures: chloroform, methanol, and triethylamine (5:2:1), R_F = 0.36; chloroform, methanol, and acetic acid (3:1:1), R_F = 0.80; and *n*-butanol, acetic acid, and water (4:1:1), R_F = 0.86.
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Acetaminophen: Potentially Toxic Metabolite Formed by Human Fetal and Adult Liver Microsomes and Isolated Fetal Liver Cells

Abstract. *A reactive metabolite of acetaminophen is hepatotoxic in humans when the drug is ingested in large overdoses. The ability of the human fetal and adult liver to oxidize acetaminophen by trapping the potentially toxic metabolite as a glutathione conjugate has been measured. Oxidation by fetal liver was approximately ten times slower than by adult liver. However, there was a definite increase in acetaminophen oxidation with fetal age. Isolated human fetal liver cells conjugated acetaminophen with sulfate but not with glucuronic acid. The results indicate that the human fetal liver is able to detoxify acetaminophen by conjugation. However, it also catalyzes the formation of an active metabolite of acetaminophen through oxidation. Hence the fetus remains at risk should a large dose of the drug cross into the fetal circulation.*

The increasing number of drugs that are transformed into reactive intermediates in the body (*1*) raises the possibility of toxic effects on the exposed human fetus. The human fetus is at special risk from such drugs because, in contrast to fetuses from animals, it is able to oxidize drugs during the first part of gestation (*2*). Active metabolite formation has been proposed as the mechanism by which overdoses of acetaminophen, a widely used analgesic and antipyretic drug, produce centrilobular liver necrosis in humans (*3*). Studies in animals have shown that acetaminophen is oxidized by a microsomal mixed-function oxidase to a reactive arylating intermediate normally detoxified by conjugation with glutathione (GSH). If liver stores of GSH become severely depleted the intermediate binds with cell macromolecules and presumably results in cell death (*4*). A high-pressure liquid chromatographic (HPLC) method has been

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developed for the quantitation of the active metabolite of acetaminophen as a GSH conjugate (*5*). We used this technique to investigate the oxidative and synthetic pathways of acetaminophen metabolism in human fetal and adult liver microsomes as well as in isolated human fetal liver cells. We now report that human fetal liver can oxidize acetaminophen to an active metabolite. Furthermore, our studies in human fetal liver cells reveal conjugation of acetaminophen with GSH and sulfate and demonstrate the utility of this preparation for drug metabolic studies.

Specimens of human adult liver from patients without cerebral activity who were selected as kidney donors were obtained within 20 minutes after artificial respiration and life-supporting treatments were stopped. Specimens of human fetal liver were obtained from fetuses that were legally aborted for sociomedical reasons (*6*). The metabolism of acetaminophen was studied in liver microsomal preparations and in human fetal liver cells (*7*) isolated by the method of Fry *et al.* (*8*). Acetaminophen metabolites were quantitated by HPLC as described previously (*9*).

Acetaminophen was oxidized by human fetal and adult liver microsomes in the presence of a nicotinamide adenine dinucleotide phosphate (NADPH) generating system to an intermediate which was trapped as a water-soluble conjugate by the addition of GSH. In the absence of an NADPH-generating system, no GSH conjugate was detected. In adult livers acetaminophen metabolism ranged from 6.5 to 21.2 nmole per milligram of microsomal protein per 10 minutes (Fig. 1a). These individual differences may be due in part to competitive interaction with drugs such as corticosteroids, which were administered in high doses before the death of some patients, and may not necessarily represent the variability in a healthy population. It should be noted that persons who attempt suicide with acetaminophen are often heavily exposed to other drugs that might influence drug metabolism. Our results are in agreement with those of Dybing (*10*), who reported that microsomes prepared from a single human adult liver metabolized acetaminophen to an intermediate that was covalently bound to microsomal protein.

Fetal liver microsomes oxidized acetaminophen to an active metabolite; however, their mean activity was approximately one-tenth that of adult liver microsomes. These results are consistent with the presence of the components of the microsomal electron transport system NADPH-cytochrome c reductase and cytochrome P-450 and confirm earlier reports on the ability of human fetal liver to metabolize xenobiotics (*2*) and drugs such as carbamazepine and desmethylimipramine (*11*). It was not possible to correlate the mothers' smoking habits (all but one were smokers) or ingestion of drugs with the ability of the fetal liver to oxidize acetaminophen. However, there was a significant correlation between the rate of formation of acetaminophen-GSH conjugate and fetal age (Fig. 1b). Whether this activity continues to increase beyond a gestational age of 23 weeks cannot be determined from the present data. Postnatally, age-related qualitative differences in metabolite excretion have been described even though the total urinary excretion rate of acetaminophen and its metabolites is similar in neonates (0 to 3 days old), young children (3 to 9 years old), and adults (*12*). Because only a negligible amount of acetaminophen is eliminated unchanged, it might be concluded that the rate of metabolism is similar in neonates and adults.

Table 1. Formation of acetaminophen conjugates in human fetal liver cells.

Incubation time (min)	Liver	Acetaminophen conjugate produced (nmole per 10^6 cells)		
		GSH	Sulfate	Glucuronide
0	1*	N.D.†	N.D.	N.D.
	2	N.D.	N.D.	N.D.
15	1			
	2	0.20	0.53	N.D.
30	1	0.14	0.72	N.D.
	2	0.20	1.04	N.D.
60	1	0.70	1.74	N.D.
	2	0.26	1.90	N.D.

*Specimens from livers 1 and 2 were obtained from fetuses of 19 and 22 weeks gestational age, respectively. The concentrations of the cells in the incubations were 7.3×10^6 per milliliter for liver 1 and 10.4×10^6 per milliliter for liver 2. †N.D., not detectable.

Isolated liver cells have definite advantages over subcellular fractions for the study of drug oxidation and conjugation reactions. The drug-metabolizing enzymes are in the presence of physiological concentrations of cofactors, and a broader range of metabolites are formed than is usually the case in subcellular enzyme preparations when cofactors need to be added. When isolated human fetal liver cells were incubated with acetaminophen (10 m*M*) the GSH and sulfate conjugates were detected (Table 1). The formation of the sulfate conjugate of acetaminophen was nearly linear with time. In contrast, the production of the GSH conjugate was not linear with time and in one experiment (liver 2) there was no increase in conjugate accumulated between 15 and 60 minutes. These data are not in agreement with findings in mouse and rat liver cells (*10*) and they might be due to a limited amount of GSH, a limited formation rate of the oxidized acetaminophen metabolite, or both. The quantitative aspects of the data presented in Table 1 are limited by the number of fetal livers available for the preparation of isolated cells. Nevertheless, our data illustrate several qualitative aspects of the use of isolated fetal liver cells for the study of drug metabolism.

Because acetaminophen does not form a GSH conjugate without prior oxidation, these data demonstrate the presence of cytochrome P-450 activity in the intact cells and confirm our results with fetal liver microsomes. Furthermore, the presence of GSH in the isolated fetal cells is established. This was confirmed by measuring GSH and acid-soluble sulfhydryl groups in fetal liver homogenates (*13*). The formation of a sulfate conjugate indicates that the enzyme sulfotransferase and the active form of sulfate, 3′-phosphoadenosine-5′-phosphosulfate (PAPS), were both present in the isolated human fetal liver cells. In normal volunteers, when large doses of acetaminophen (within the therapeutic range) were administered, there was saturation of sulfate conjugation (*14*). The dose level

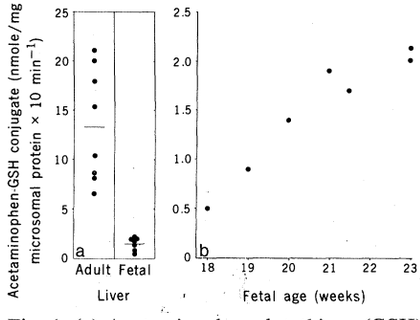

Fig. 1. (a) Acetaminophen-glutathione (GSH) conjugate formation by human adult ($N = 8$) and fetal liver ($N = 8$) microsomes. Initial concentrations of acetaminophen and GSH were 10 and 5 m*M*, respectively. Kinetic analysis of the data from five adult and four fetal livers revealed the following apparent values. For the adult liver the rate $V_{max} = 8.5 \pm 3.5$ nmole per milligram of microsomal protein per 10 minutes (mean ± standard deviation) and the Michaelis constant $K_m = 1.2 \pm 1.3$ m*M*; for the fetal liver $V_{max} = 1.2 \pm 0.5$ nmole/mg per 10 minutes and $K_m = 0.8 \pm 0.5$ m*M*. (b) Correlation of acetaminophen-GSH conjugate formation in human fetal liver ($N = 7$) with gestational age estimated by crown-to-rump length (correlation coefficient $r = 0.96$) (age was not estimated in one fetus). Initial acetaminophen and GSH concentrations were the same as in (a).

at which this might occur in the fetus is not known.

Glucuronidation of acetaminophen in isolated fetal liver cells was not detectable (Table 1). Attempts to demonstrate glucuronidation in human fetal liver microsomes have revealed negligible activity compared to the adult (*15*), which might be ascribed to low concentrations of both uridine diphosphate glucuronic acid (UDPGA) and microsomal glucuronyl transferase. Glucuronidation of many drugs and endogenous substrates is deficient (*16*) or poorly developed in the neonate (*17*). Moreover, sulfation of acetaminophen predominates over glucuronidation in the neonate (*12*). The presence of a sulfate but not a glucuronide conjugate of acetaminophen in human isolated fetal liver cells is in agreement with all these data.

Our results do not suggest that acetaminophen is unsafe during pregnancy when ingested in therapeutic doses for short periods of time. The fetal liver apparently has the ability to detoxify acetaminophen by conjugation with sulfate and GSH. The results do demonstrate that the fetal liver can oxidize acetaminophen to an active metabolite. It is not known whether the quantity of the drug that passes into the fetal circulation when the mother has taken a sublethal dose is large enough to result in damage to the fetal liver, depletion of GSH, and saturation of sulfate conjugation.

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6. The study was approved by the Ethical Committee of the Karolinska Institute and the Swedish National Board of Health and Welfare.
7. Liver microsomes were incubated at a protein concentration of 2 mg/ml in the presence of an NADPH-generating system, GSH (5 m*M*), and acetaminophen (10 m*M*) at 37°C. The reaction was stopped by addition of 3*M* perchloric acid, the mixture was centrifuged, and the supernatant was stored frozen at −20°C until analysis. Formation of the acetaminophen-GSH conjugate by human fetal and adult liver microsomes was linear with time up to 20 minutes and with a microsomal protein concentration up to 4 mg/ml. Human fetal liver cells were isolated by the method of Fry *et al.* (*8*) for rat liver cells with the modification that the cells were allowed to sediment by gravity. Cell viability was examined by exclusion of trypan blue. Viable cells were counted in a Bücher chamber. More than 90 percent of the cells excluded trypan blue. Ultrastructural analysis substantiated the exclusion dye test in demonstrating general criteria for viable cells without signs of anoxic degeneration. Isolated fetal liver cells (7 to 10 × 10^6 cell/ml) in 10 ml of Eagle minimum essential medium (Bio-Cult, Scotland) containing 10 percent fetal calf serum, penicillin (100 unit/ml), streptomycin (100 μg/ml), and acetaminophen (10 m*M*) were incubated at 37°C. At incubation times from 15 to 60 minutes 1 ml of the incubate was removed and handled as described above.
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Angiogenesis in the Mouse Cornea

Abstract. *We have developed a method that permits analysis of neovascular responses in the mouse cornea. Using this method we have demonstrated that both allogeneic lymphocytes and a variety of tumors can induce angiogenesis, but that only the latter appear capable of eliciting secondary capillary sprouting.*

The importance of angiogenesis, or the formation of new blood vessels, is well recognized in a variety of disease processes, in inflammatory reactions, during the course of graft-versus-host reactions, in delayed hypersensitivity responses, and most dramatically as an essential component of tumor growth (*1*). Yet our understanding of the mechanisms underlying such induced neovascularization is limited, largely because no test systems have been developed that permit a critical assessment of the various component contributors to the angiogenesis phenomenon.

The primary test systems for angiogenesis in vivo are neovascular responses induced on the chicken embryo chorioallantoic membrane (CAM) (*2*) and neovascularization induced by im-

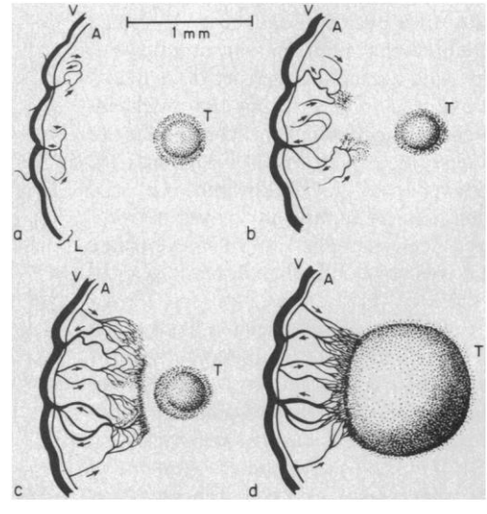

Fig. 1. A sequence of vascular responses in the cornea of albino C57BL/6 mice after implantation of C755 mammary adenocarcinoma fragments. The drawings of the living cornea were made under a stereomicroscope at (a) 2 days, (b) 6 days, (c) 8 days, and (d) 12 days after grafting. Vein (*V*) and artery (*A*) of the limbus (*L*) are shown in relation to the tumor graft (*T*). Arrows indicate the direction of blood flow.

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