very little inhibition (10 to 15 percent) at concentrations of dibutyryl cyclic AMP as high as $10^{-4}M$. Although the cells used in the experiment described in Table 2 were trypsinized from stock cells with 0.25 percent trypsin, another experiment comparing 0.25 percent trypsin in citrate saline with 0.05 percent trypsin in 0.5 mM Na_2EDTA (*4*) also showed no killing by 10^{-8}, 10^{-6}, and $10^{-4}M$ dibutyryl cyclic AMP.

We know of no other evidence showing that human fibroblasts are killed by glucocorticoids. The nonhuman fibroblast cell line most studied in this respect is the mouse L cell. However, whereas steroids inhibit the growth rate of these cells, they do not cause the cytolysis that occurs in some nonfibroblast cell types, such as thymic lymphocytes (*18*).

Other investigators have found some stimulation of fibroblast growth by glucocorticoids (*19, 20*), some have found no effect (*21, 22*), and one group reported some inhibition with $3 \times 10^{-6}M$ cortisol and prednisolone (*23*). Milo *et al.* (*24*) showed that estradiol and cortisol at concentrations as high as $2 \times 10^{-6}M$ did not inhibit cloning of human embryonic lung and newborn foreskin fibroblasts; in fact, glucocorticoids, under certain conditions, are required for best clonal growth (*24, 25*).

An entire field of well-documented research is concerned with the lengthening of the lifetime in vitro of human embryonic lung fibroblasts by $10^{-5}M$ cortisone and cortisol (*26-28*). Although the lifetimes in vitro of skin fibroblasts are not uniformly lengthened by glucocorticoids, no large inhibitions of cell growth are observed (*29*). In particular, strain GM316, which both we and Breslow *et al.* (*2*) have tested, was found to be unaffected (*29*).

Thus, as discussed by Grove *et al.* (*29*), fibroblasts may vary in their response to glucocorticoids depending on the tissue source and the age of the donor. In addition, skin fibroblasts may differ according to the type of skin and the microenvironment of the particular sample. However, killing or large inhibition of growth is not seen even in the continual presence of steroids; thus the observation by Epstein and his co-workers (*1-3*) that exposure to low doses of dexamethasone for 24 hours causes significant killing of human skin fibroblasts is unique. When we measure the effect of dexamethasone, dibutyryl cyclic AMP, and ouabain in straightforward cloning assays performed independently, we observe a killing effect by ouabain only. Thus, there appears to be no basis for the diagnosis of CF in homozygotes or heterozygotes by a technique based on a differential kill by dexamethasone of their fibroblasts compared to normal fibroblasts (*2*).

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7. Media powder, Hanks salts, and trypsin were from Gibco; pyruvate, dexamethasone, and Hepes buffer were from Sigma; asparagine and serine were from Calbiochem; glucose, sodium citrate, and potassium chloride were from Baker; sodium chloride, dimethyl sulfoxide, and Giemsa stain were from Fisher; ouabain was from Lilly; penicillin G was from Parke, Davis; and streptomycin was from Pfizer. The dexamethasone phosphate was a gift from Merck Sharp & Dohme. Fetal bovine serum was from both Microbiological Associates and Gibco.
8. Since medium containing 10 percent serum blocks transmission, we dissolved dexamethasone phosphate to $10^{-3}M$ in medium plus 10 percent fetal calf serum, filtered it to sterilize, and diluted it with Hepes-buffered Eagle's MEM (1:9) to give an absorbance of 2.736 at 239 nm compared to the control (without dexamethasone) absorbance of 1.246. Thus the chemical dissolves easily in medium and passes through a 0.22-μm filter in the presence of 10 percent serum.
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13. Use of a minimum of 60 cells per colony (as done by Epstein and his co-workers) does not unmask any effect of dexamethasone. Besides using this higher minimum in three experiments, we also reinspected the fixed dishes that were used to grow strains GM1582, GM316, and GM498 (Table 1). Values for cloning efficiency decreased 2 to 5 percent, but the survival percentage showed no change from that given in Table 1.
14. Our differences with Epstein and his co-workers do not arise from the use of different cell strains, since we have tested at least nine normal cell strains. For GM316 they observed 51 percent survival at $10^{-10}M$ dexamethasone phosphate, decreasing to 10 percent at $10^{-6}M$; for GM1582 their values were 57 and 22 percent at 10^{-10} and $10^{-6}M$ dexamethasone phosphate, respectively (*2*). Our values for these strains are given in Table 1.
15. The cultures were fed at 8 days and fixed at 13 days; colonies of more than about 60 cells were counted on five dishes for each condition by three observers. Each observer reported a steady decrease in cloning efficiency with increasing size of the inoculum.
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30. The experiments in Toronto were performed by A. Barkas and L. Wijcik and were supported by the Canadian Cystic Fibrosis Foundation and the Sellers Foundation. J.B.K. was supported by grant MY402 from the U.S. Cystic Fibrosis Foundation.

22 August 1979

Metabolism of Theophylline to Caffeine in Human Fetal Liver

Abstract. *Caffeine (1,3,7-trimethylxanthine) is a biotransformation product of theophylline (1,3-dimethylxanthine) in the human fetus. Liver explants, obtained from human fetuses with gestational ages of 12 to 20 weeks, were incubated with theophylline and produced caffeine and, in lesser amounts, 1,3-dimethyluric acid and 3-methylxanthine. These findings suggest that the predominant pathway in theophylline metabolism in the fetus and newborn infant is the methylation reaction producing caffeine. This may contribute to the neonate's exceedingly slower elimination of caffeine relative to theophylline. Caffeine produced from theophylline may add to the pharmacologic effects of theophylline in newborn infants with apnea.*

Theophylline (1,3-dimethylxanthine), an active alkaloid from tea, is a potent central nervous system and cardiovascular stimulant with diuretic and bronchial smooth-muscle relaxant properties. Recently, this drug was shown to be effective in the prevention and treatment of apnea in the premature newborn infant (*1*); indeed, it has gained increasing acceptance as the major pharmacotherapeutic agent for the management of neonatal apnea. Despite its widespread use, however, little is known about the biotransformation of this drug in the newborn infant. In adults, the major metabolic pathway involves demethylation and oxidation reactions producing monomethylxanthines and methyluric

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Table 1. Biotransformation of theophylline to caffeine in human fetal liver cultures.

Fetal age (weeks)	Fetal size, crown to rump (mm)	Total protein* (mg per incubation mixture	Caffeine formed* (nmole/mg protein per 24 hours)
12.5	90	0.80	0.23
12.5	75	0.34	0.86
13.0	90	0.60	1.98
14.5	110	0.37	1.01
17.5	152	0.80	2.06
20	185	0.61	2.36
	Mean ± standard error		
15.00 ± 1.3	117.00 ± 17.4	0.59 ± 0.08	1.42 ± 0.34

*Individual values are means of more than six determinations.

acids. Theophylline is metabolized to 1-methyluric acid, 1,3-dimethyluric acid, and 3-methylxanthine (*2, 3*).

In the premature newborn infant with apnea, administration of theophylline results in increasing concentrations of both theophylline and caffeine in the plasma (*4*). In contrast, adults given theophylline do not produce caffeine (*5*); rather, caffeine is metabolized to form theophylline (*6*). These findings suggest that the fetus and newborn infant may utilize methylation pathways to metabolize theophylline. Our report demonstrates that the human fetal liver is capable of methylating theophylline to caffeine as early as the 12th week of gestation, and that the methylation pathway is the predominant reaction in theophylline metabolism in this age group.

Liver explants were obtained from six human fetuses aborted at 12 to 20 weeks of gestation. (These abortions were reviewed and approved by the Committee of Medical and Dental Acts of the Royal Victoria Hospital and by the Montreal General Hospital.) The explants (1 to 2 mm) were incubated five per plate in a modified Leibovitz medium (*7*). The viability of this preparation for 10 days has been demonstrated with morphological and biochemical techniques (*7*). After incubation for 24 hours, the explants were allowed to equilibrate for 48 hours. The medium was then replaced with Leibovitz medium containing theophylline (1 m*M*), ^{3}H-labeled *S*-adenosyl methionine (1 μCi; specific activity, 1 μCi per micromole of unlabeled *S*-adenosyl methionine) as the methyl donor, $MgCl_2$ (5 m*M*), and glutathione (5 m*M*). The explants were then incubated for 24 to 72 hours. Control plates contained the same nutrient media and reaction mixtures, but no fetal liver explants. At various intervals, the media and the explants were removed and caffeine, theophylline, and other metabolites in the media were determined by using high-pressure liquid chromatography and thin-layer chromatography. The medium (2 ml) was acidified with 1*N* HCl (0.1 ml) and extracted twice with chloroform. Cold water-saturated chloroform (5 ml) was added and mixed to the medium, followed by centrifugation. The organic phase was removed, the extraction with chloroform was repeated, and the two organic phases were pooled. The organic phase was evaporated to dryness; the residue was redissolved in chloroform (50 μl) and applied to a fluorescent thin-layer chromatographic plate (silica gel G) with a 15-cm solvent front. The solvent contained chloroform, *n*-butanol, acetone, and NH_4OH at proportions of 40:30:40:10, respectively. This solvent allowed easy distinction of caffeine from theophylline with R_F values of 0.70 and 0.18, respectively.

Figure 1 shows the incorporation of the methyl group into theophylline to form caffeine as a function of time. After a lag phase of 4 to 8 hours, the rate of caffeine production was linear until about 52 hours, with a mean production of 1.25 nmole of caffeine per milligram of protein every 24 hours. In the control plate (no fetal liver present), no caffeine formation was measured (Fig. 1). The slow rate of theophylline biotransformation in vitro suggests a distinct advantage of this organ culture system relative to the isolated liver microsomal preparation (*8, 9*). Studies of theophylline and caffeine metabolism in which liver homogenates and microsomal preparations from neonatal and adult rats were used failed to show significant methylation during a 1-hour incubation (*10*).

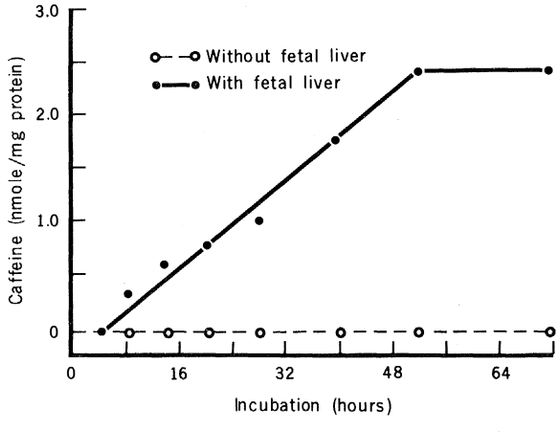

Fig. 1. Methylation of theophylline to caffeine in human fetal liver culture. The finding that there was no caffeine production in the absence of liver tissue (broken line) eliminates the possibility of spontaneous methylation of theophylline. Control plates containing liver tissue but no theophylline also showed no caffeine production.

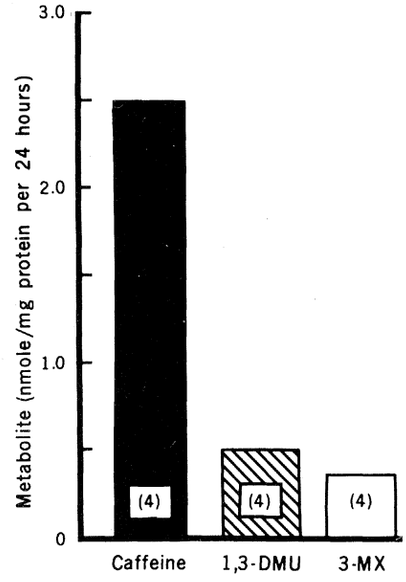

Fig. 2. Metabolism of theophylline by the human fetal liver in vitro, showing formation of caffeine, 1,3-dimethyluric acid (*1,3-DMU*), and 3-methylxanthine (*3-MX*). There were four determinations in each case.

Table 1 shows the biotransformation of theophylline to caffeine in the fetal liver culture. In the age interval studied (12.5 to 20 weeks), theophylline methyltransferase activity ranged from 0.23 to 2.36 nmole per milligram of protein every 24 hours. These values are considerably lower than the methyltransferase activities specific for sulfur-containing amino acids from the human fetal brain, liver, and kidney (*11*). These observations could reflect differences in the specificity of methyltransferase activities or differences in the experimental technique (liver culture versus liver homogenates) (*11*). Increasing rates of caffeine formation seem to appear with increasing fetal age (Table 1); however, the correlation was not significant ($P = .08$, $r = .75$).

We examined the possibility that oxidative metabolism of theophylline occurred simultaneously with the methylation to caffeine. A high-pressure liquid chromatographic analysis of the media

(*12*) showed that 1,3-dimethyluric acid and 3-methylxanthines were produced in addition to caffeine (Fig. 2). However, the amount of caffeine produced was five times greater than the oxidative metabolites, suggesting that the predominant pathway of theophylline metabolism in the human fetus is methylation.

The presence of active methylation reactions (*11, 13, 14*) and the low activity of the hepatic microsomal oxidative enzymes in the human fetus and newborn infant (*15*) helps to explain the production of caffeine in neonates that are given theophylline (*4*). Our report provides evidence that caffeine is not only produced by the fetal liver but could be the major metabolite of theophylline.

It is intriguing to relate these results to the exceedingly slow elimination of caffeine in the newborn infant (mean half-life in plasma, 102 hours compared to 6 hours in nonsmoking adults) (*16, 17*) and to the faster elimination of theophylline in the neonate (mean half-life in plasma, 30 hours; adult value, 6 hours) (*1, 18*). The continued methylation of the dimethylxanthines to caffeine with negligible demethylation or C_8 oxidation of caffeine (*12, 19*) could result in caffeine's accumulation in plasma and slower elimination in the premature infant (*16*).

It is possible that theophylline acquired transplacentally, as when the mother drinks tea, may contribute to the fetal (and neonatal) caffeine load (*5, 20*). Direct administration of theophylline to newborn infants with apnea results in concentrations of caffeine in plasma that can elicit significant pharmacotherapeutic activity (*1*). Thus, the pharmacologic effects in infants treated with theophylline may in part be contributed by caffeine.

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Choline Excites Cortical Neurons

Abstract. *In cats under halothane or methoxyflurane, iontophoretic applications of choline are only eight times weaker than applications of acetylcholine in evoking firing of neurons in the sensorimotor region of the cerebral cortex. The action of choline is suppressed by atropine but not by two agents that block choline uptake (hemicholinium-3 and triethylcholine), and is not potentiated by an anticholinesterase (physostigmine). Choline therefore appears to excite cortical neurons by a direct action, which may be a significant component of its beneficial therapeutic effects.*

As a precursor of acetylcholine (ACh), choline is an essential factor in cholinergic transmission (*1*). According to several recent reports (*2*), some patients suffering from tardive dyskinesia and possibly other disorders are significantly improved by ingestion of relatively large doses of choline or lecithin (from which choline is freed after absorption). These patients thus appear to suffer from a deficiency in cholinergic transmission in the brain, and the beneficial action of choline is thought to be mediated by an increase in neuronal ACh available for release from cholinergic nerve endings (*3, 4*). Another possible mechanism must be kept in mind. Choline is itself an ACh agonist (*5*), and therefore large doses of choline, which are known to raise greatly choline levels in the brain (*3, 4*), might have a significant direct action on cerebral neurons. In our first systematic investigation of cortical cholinoceptive neurons (*6-8*), choline was reported to be a weak excitant of ACh-sensitive cells. In view of the present great interest in choline and its clinical use (*9*), it seemed important to reinvestigate this topic in more detail. Judging by the relative intensities of equipotent iontophoretic currents, choline appears to be quite a strong excitant of ACh-excitable neurons, being only some eight times weaker than ACh itself.

Multibarrel glass micropipettes (*10*) were inserted into the pericruciate cortex of cats under light gaseous anesthesia (*11*). Under these conditions, neurons that are situated in the deeper cortical layers and discharge spontaneously in a characteristic manner (*6*) are particularly sensitive to ACh; as shown by the record of firing frequency of such a neuron in Fig. 1, these cells are often strongly excited by iontophoretic applications of 14 nA or less [probably equivalent to release of < 50 fmole/sec (*12*)]. When choline was released from another barrel of the micropipette, a response was obtained that was comparable in both intensity and time course (note especially the prolonged aftereffect), but a greater application of choline was almost always necessary: in Fig. 1, equipotent iontophoretic currents differed by a factor of 10 (it is also evident from Fig. 1 that the excitant action of choline compares quite favorably with that of glutamate released by an identical but briefer iontophoretic current). The mean ratio of

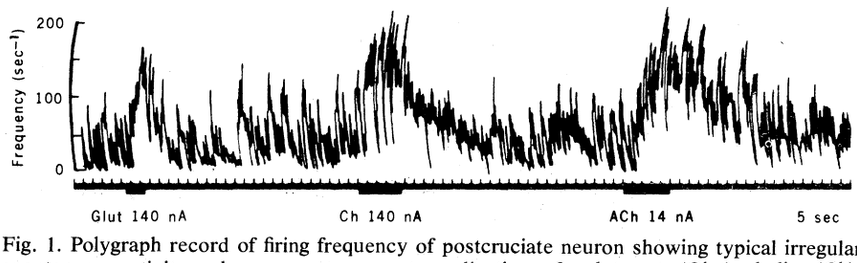

Fig. 1. Polygraph record of firing frequency of postcruciate neuron showing typical irregular spontaneous activity and responses to separate applications of L-glutamate (*Glut*), choline (*Ch*), and acetylcholine (*Ach*). Intensity and duration of microiontophoretic applications are indicated.

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