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April 25, 2000

Dr. Oscar Hernandez
US EPA/OPPT/CCD (7405)
401 M Street, SW
Room E615B
Washington, DC 20460

VIA FEDERAL EXPRESS

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Dear Dr. Hernandez:

Enclosed are copies of the following documents which were inadvertently omitted from our package sent to you last Friday, April 21, 2000:

1. Protocol 418-008 Combined oral (gavage) fertility, developmental and perinatal/postnatal reproduction toxicity study of PFOS in rats (Sponsor's study number: 6295.9), Table E20, 1-3;

2. Harb, R.V., Hartman, H.A. and Cox, R.H. (1994), Prevention of fluvastatin induced toxicity, mortality, and cardiac myopathy in pregnant rates by mevalonic acid supplementation. *Teratology*, **50**, 19-26;

3. Haughom, B. and Spydevold, O. (1992), The mechanism underlying the hyperlipemic effect of perfluorooctanoic acid (PFOA), perfluorooctate sulphonic acid (PFOS) and clofibric acid. *Biochimica et Biophysica Acta,* **1128**, 65-72;

4. Levin, M.S. Pitt, A.J.A., Schwartz, A.L., Edwards, P.A. and Gordon, J.I. (1989), Developmental changes in the expression of genes involved in cholesterol biosynthesis and lipid transport in human and rat fetal and neonatal livers. *Biochimica et Biophysica Acta,* **1003**, 293-300; and

5. Interoffice memo from Tom Kestner to Leo Gehlhoff, "Fluorochemical Isomer Distribution by ^{19}F-NMR Spectroscopy," December 1, 1997.

If you have any questions or need additional information, please feel free to contact me at 651-733-5181.

Sincerely,

LRZobel MD

Larry R. Zobel, MD MPH
Staff Vice President & Medical Director

LRZ:kmj
Enclosures

PROTOCOL 418-008: COMBINED ORAL (GAVAGE) FERTILITY, DEVELOPMENTAL AND PERINATAL/POSTNATAL REPRODUCTION TOXICITY STUDY OF PFOS IN RATS (SPONSOR'S STUDY NUMBER: 6295.9)

TABLE E20 (PAGE 1): LITTER OBSERVATIONS (NATURALLY DELIVERED PUPS) - SUMMARY - F2 GENERATION LITTERS

MATERNAL DOSAGE GROUP		I	II	III
MATERNAL DOSAGE (MG/KG/DAY)		0 (VEHICLE)	0.1	0.4
DELIVERED LITTERS WITH ONE OR MORE LIVEBORN PUPS	N	22	21	24
PUPS DELIVERED (TOTAL)	N	316	325	357
	MEAN±S.D.	14.4 ± 3.5	15.5 ± 2.5	14.9 ± 2.6
LIVEBORN	MEAN±S.D.	14.1 ± 3.5	15.1 ± 2.4	14.3 ± 2.5
	N(%)	310(98.1)	318(97.8)	344(96.4)
STILLBORN	MEAN±S.D.	0.3 ± 0.6	0.3 ± 1.1	0.5 ± 0.9
	N(%)	6(1.9)	7(2.2)	13(3.6)
PUPS FOUND DEAD OR PRESUMED CANNIBALIZED				
DAY 1	N/N(%)	2/310(0.6)	2/318(0.6)	2/344(0.6)
DAYS 2- 4	N/N(%)	7/308(2.3)	2/316(0.6)	11/342(3.2)
DAYS 5- 7	N/N(%)	0/171(0.0)	2/167(1.2)	5/191(2.6)
DAYS 8-14	N/N(%)	0/171(0.0)	1/165(0.6)	1/178(0.6)a
DAYS 15-21	N/N(%)	0/171(0.0)	0/164(0.0)	0/177(0.0)a
VIABILITY INDEX b	%	97.1	98.7	96.2
	N/N	301/310	314/318	331/344
LACTATION INDEX c	%	100.0	98.2	96.7
	N/N	171/171	164/167	177/183

DAY(S) = DAY(S) POSTPARTUM

a. Excludes values for litter 13257; the dam was found dead on day 10 of lactation; eight pups were sacrificed.

b. Number of live pups on day 4 (preculling) postpartum/number of liveborn pups on day 1 postpartum.

c. Number of live pups on day 21 (weaning) postpartum/number of live pups on day 4 (postculling) postpartum.

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TABLE E20 (PAGE 2): LITTER OBSERVATIONS (NATURALLY DELIVERED PUPS) - SUMMARY - F2 GENERATION LITTERS

MATERNAL DOSAGE GROUP MATERNAL DOSAGE (MG/KG/DAY)		I 0 (VEHICLE)	II 0.1	III 0.4
DELIVERED LITTERS WITH ONE OR MORE LIVEBORN PUPS	N	22	21	24
SURVIVING PUPS/LITTER a				
DAY 1 b	MEAN±S.D.	14.1 ± 3.5	15.1 ± 2.4	14.3 ± 2.5
DAY 4 PRECULLING	MEAN±S.D.	13.7 ± 3.3	15.0 ± 2.8	13.8 ± 2.4
DAY 4 POSTCULLING	MEAN±S.D.	7.8 ± 0.8	8.0 ± 0.2	8.0 ± 0.2
DAY 7	MEAN±S.D.	7.8 ± 0.8	7.8 ± 0.5	7.8 ± 0.7
DAY 14	MEAN±S.D.	7.8 ± 0.8	7.8 ± 0.7	7.7 ± 0.8 [24]c
DAY 21	MEAN±S.D.	7.8 ± 0.8	7.8 ± 0.7	7.7 ± 0.8 [24]c
PERCENT MALE PUPS PER NUMBER OF PUPS SEXED				
DAY 1 b	MEAN±S.D.	47.9 ± 15.2	52.1 ± 10.9	47.0 ± 13.3
DAY 4 PRECULLING	MEAN±S.D.	47.9 ± 15.1	51.5 ± 11.1	46.6 ± 13.4
DAY 4 POSTCULLING	MEAN±S.D.	47.0 ± 10.0	49.6 ± 1.6	49.1 ± 5.7
DAY 7	MEAN±S.D.	47.0 ± 10.0	48.9 ± 3.9	48.7 ± 6.4
DAY 14	MEAN±S.D.	47.0 ± 10.0	49.2 ± 2.6	48.3 ± 6.2 [24]c
DAY 21	MEAN±S.D.	47.0 ± 10.0	49.2 ± 2.6	48.3 ± 6.2 [24]c

DAY = DAY POSTPARTUM
[ ] = NUMBER OF VALUES AVERAGED
a. Average number of live pups per litter, including litters with no surviving pups.
b. Includes pups born alive, found dead day 1 postpartum.
c. Excludes values for litter 13257; the dam was found dead on day 10 of lactation; eight pups were sacrificed.

PROTOCOL 418-008: COMBINED ORAL (GAVAGE) FERTILITY, DEVELOPMENTAL AND PERINATAL/POSTNATAL REPRODUCTION TOXICITY STUDY OF PFOS IN RATS (SPONSOR'S STUDY NUMBER: 6295.9)

TABLE E20 (PAGE 3): LITTER OBSERVATIONS (NATURALLY DELIVERED PUPS) - SUMMARY - F2 GENERATION LITTERS

MATERNAL DOSAGE GROUP		I	II	III
MATERNAL DOSAGE (MG/KG/DAY)		0 (VEHICLE)	0.1	0.4
DELIVERED LITTERS WITH ONE OR MORE LIVEBORN PUPS	N	22	21	24
LIVE LITTER SIZE AT WEIGHING				
DAY 1	MEAN±S.D.	14.0 ± 3.5	15.0 ± 2.6	14.2 ± 2.5
DAY 4 PRECULLING	MEAN±S.D.	13.7 ± 3.3	15.0 ± 2.8	13.8 ± 2.4
DAY 4 POSTCULLING	MEAN±S.D.	7.8 ± 0.8	8.0 ± 0.2	7.9 ± 0.3
DAY 7	MEAN±S.D.	7.8 ± 0.8	7.8 ± 0.5	7.8 ± 0.7
DAY 14	MEAN±S.D.	7.8 ± 0.8	7.8 ± 0.7	7.7 ± 0.8 [23]a
DAY 21	MEAN±S.D.	7.8 ± 0.8	7.8 ± 0.7	7.7 ± 0.8 [23]a
PUP WEIGHT/LITTER (GRAMS)				
DAY 1	MEAN±S.D.	6.3 ± 0.8	6.1 ± 0.5	6.2 ± 0.5
DAY 4 PRECULLING	MEAN±S.D.	8.7 ± 1.6	8.2 ± 1.0	8.0 ± 1.3
DAY 4 POSTCULLING	MEAN±S.D.	8.8 ± 1.6	8.3 ± 1.0	8.0 ± 1.3
DAY 7	MEAN±S.D.	14.7 ± 2.4	13.9 ± 2.2	12.8 ± 2.6*
DAY 14	MEAN±S.D.	32.0 ± 3.5	31.8 ± 3.1	28.9 ± 4.7** [23]a
DAY 21	MEAN±S.D.	50.1 ± 5.1	49.2 ± 5.0	46.5 ± 6.3 [23]a

DAY = DAY POSTPARTUM
[ ] = NUMBER OF VALUES AVERAGED
a. Excludes values for litter 13257; the dam was found dead on day 10 of lactation; eight pups were sacrificed.
* Significantly different from the vehicle control group value ($p \leq 0.05$).
** Significantly different from the vehicle control group value ($p \leq 0.01$).

From Donna 12/1/99 ** my copy *

TERATOLOGY 50:19–26 (1994)

Prevention of Fluvastatin-Induced Toxicity, Mortality, and Cardiac Myopathy in Pregnant Rats by Mevalonic Acid Supplementation

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ABSTRACT Mevalonic acid is a product of the enzyme HMG-CoA reductase which is essential for cholesterol biosynthesis. Fluvastatin (Sandoz compound XU 62-320) is a potent inhibitor of this enzyme and, hence, mevalonic acid production. In three separate studies, oral administration of fluvastatin at 12 and 24 mg/kg/day to mated rats from day 15 of gestation through weaning resulted in unanticipated maternal mortality at the time of parturition and during lactation. Microscopic evaluations performed in two studies revealed significant cardiac myopathy in the dying animals. Drug-related clinical signs, significant maternal body weight loss, and an increase in stillborn pups and neonatal mortality were also noted at one or both dose levels. Supplementation of fluvastatin administration with 500 mg/kg b.i.d. of mevalonic acid completely blocked and/or ameliorated the mortality, cardiac myopathy, and other adverse effects. These studies indicate that the adverse maternal effects observed with fluvastatin before or following parturition resulted from exaggerated pharmacologic activity at the dose levels administered, i.e., inhibition of the enzyme HMG-CoA reductase, its immediate product mevalonic acid, and cholesterol biosynthesis.
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? cardiac myopathy

Fluvastatin (Sandoz compound XU 62-320) is a synthetic potent inhibitor of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis (Engstrom et al., '88; Kathawala et al., '88; Kathawala, '91). Administration of fluvastatin in rats, dogs, and monkeys induces significant reductions in serum total cholesterol, low-density lipoprotein cholesterol, and serum triglyceride levels (Engstrom et al., '88). During the safety assessment of fluvastatin, nonclinical studies were performed to evaluate the effect of this compound on fertility, reproductive performance, and teratogenicity in rats and rabbits. There were no adverse effects on fertility or reproductive performance up to the highest dose levels tested in male (20 mg/kg/day) and female (6 mg/kg/day) rats, and no evidence of teratogenic activity in rats at doses up to 36 mg/kg/day or rabbits at doses up to 10 mg/kg/day (R.V. Hrab, unpublished data). However, in a perinatal/postnatal study (Segment III), 12 and 24 mg/kg/day of fluvastatin administered to pregnant rats from day 15 pc (post coitus) through weaning resulted in maternal mortality at or near term and during the postpartum period. This mortality was replicated in a subsequent follow-up study, and microscopic tissue evaluation revealed the occurrence of cardiomyopathy only in the dying animals. No cardiac pathology was detected in nonpregnant animals in these studies with fluvastatin. As in previous rat studies (Stoll et al., '88), forestomach epithelial hyperplasia and hyperkeratosis were found. These have been shown to be specific to rodents and to be a result of contact irritation by fluvastatin (Robison et al., '94).

Coadministration of mevalonic acid, the immediate product of the enzyme HMG-CoA reductase, has been shown to prevent or antagonize various organ toxicities induced in rats and rabbits with other HMG-CoA reductase inhibitors (MacDonald et al., '88; Kornbrust et al., '89). In pregnant rats, it has been shown that 500 mg/kg b.i.d. of mevalonic acid suppressed the teratogenicity of mevinolinic acid, an inhibitor of HMG-CoA reductase, when administered prior to and approximately 5 hr after administration of mevinolinic acid (Minsker et al., '83). Since fluvastatin is an inhibitor of the enzyme HMG-CoA reductase and therefore of mevalonic acid production, a relationship was considered to exist between the exaggerated pharmacologic activity associated with HMG-CoA reductase inhibition by fluvastatin and the occurrence of maternal mortality and/or cardiac lesions. The study reported herein was designed to determine whether coadministration of me-

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This paper was submitted for publication shortly after the untimely death of Dr. Cox in August 1993.

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TABLE 1. Daily dosing schedule

Group	First dose administration	Second dose administration (approx. 1/2 hr. after first dose)	Third dose administration (approx. 5 hr. after second dose)
I	Deionized H_2O	1% CMC	Deionized H_2O
II	Mevalonic acid 500 mg/kg	1% CMC	Mevalonic acid 500 mg/kg
III	Mevalonic acid 500 mg/kg	Fluvastatin 12 mg/kg/day	Mevalonic acid 500 mg/kg
IV	Mevalonic acid 500 mg/kg	Fluvastatin 24 mg/kg/day	Mevalonic acid 500 mg/kg
V	—	Fluvastatin 12 mg/kg/day	—
VI	—	Fluvastatin 24 mg/kg/day	—

valonic acid with fluvastatin from day 15 of gestation through day 21 postpartum could inhibit or block the adverse effects noted with fluvastatin.

MATERIALS AND METHODS

Animals

One hundred twenty female rats (Charles River CD®-Sprague-Dawley-derived) obtained from Charles River Breeding Laboratories, Inc., Kingston, New York, were approximately 14 wk old at the start of the study. A group of males of the same strain and age, and from the same supplier, was used for mating, during which each female was housed with one male. Observation of vaginal plugs the following morning was considered evidence of mating and day 0 of gestation or day 0 post coitus (pc). The day on which pups were born was designated day 0 postpartum (pp), while day 21 pp was the day of weaning. As the females were mated, they were randomly assigned to six groups until 20 rats per group were mated. After mating, females were housed individually in suspended stainless steel wire-bottom cages in a temperature and humidity controlled room with a 12-hr light and 12-hr dark cycle. On day 20 of gestation, the rats were transferred to transparent plastic cages with bedding consisting of hardwood chips. Certified Purina rodent chow (pellets) and water (provided via automatic watering device) were supplied ad libitum.

Compounds administered

Lot 28 of fluvastatin (XU 62-320) was used in this experiment. Purity was determined by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) to be 99+%. DL-mevalonic acid lactone was obtained from Fluka Chemical Corp. (Ronkonkoma, NY), and purity was found to be 96+% as determined by HPLC.

where get Fluvastatin?

Dosage administration

Dosing solutions of fluvastatin were prepared fresh daily in 1% carboxymethylcellulose (CMC). Mevalonic acid was prepared fresh twice daily in deionized water. Individual animal doses were calculated and administered daily (to females only) via gavage from day 15 of gestation through day 21 postpartum at a volume of 10 ml/kg (Table 1). Dose calculations were based on the daily body weight from day 15 of gestation until delivery (or day 28 pc for those animals which did not deliver). Following delivery (day 0 pp) through weaning (day 21 pp), the dose calculations were based on the most recently recorded body weight. Control rats received three separate doses: 10 ml/kg b.i.d. of deionized water and 10 ml/kg/day of 1% CMC, also based on the same body weight schedules as in the dose groups. Concentrations of the dosing solutions were assayed and verified four times during the study.

Examinations

Individual clinical observations were recorded at least once daily. Body weights were recorded on days 0, 7, and daily from days 15 through 20 of pregnancy. After each animal delivered, maternal body weights were recorded on days 0, 7, 14, and 21 pp. Food consumption was recorded only during gestation on days 0, 7, 15, and 20 pc.

Following delivery of each litter, viability, clinical signs, and external examinations of pups were recorded on days 0 pp through 21 pp. The sex and individual weights of all viable pups were recorded on days 0, 1, 4, 7, 14, and 21 pp. Pups stillborn or found dead during the postpartum period and not autolyzed or cannibalized were examined, identified, and preserved in neutral buffered 10% formalin. Dams dying spontaneously or sacrificed moribund were necropsied. Animals which did not deliver were necropsied on day 28 pc. On day 21 pp, all remaining dams and pups were euthanized, necropsied, and examined. Pups with lesions as well as several control group pups were identified and saved in neutral buffered 10% formalin. All other pups were discarded.

Post mortem examinations

Following induction of deep surgical anesthesia using excess CO_2, animals were euthanized by severing the axillary vessels for exsanguination. Terminal body weight was recorded for each dam and a thorough dissection was performed on all surviving dams at weaning (day 21 pp). All gross lesions and approximately 40 representative tissue specimens were collected and preserved in neutral buffered 10% formalin.

Spontaneously dying animals were dissected and a complete set of tissue specimens, including the gravid reproductive tracts, were collected and preserved. Nonpregnant animals were sacrificed on day 28 pc. Since the treatment-related cardiac findings occurred only in

TABLE 2. Cause of death of spontaneously dying animals

Dose group	Total number of females that died on study	Pregnant females that died before delivery on day 22 pc	Pregnant females that died postpartum	Nonpregnant females dying during experiment
I Control	0	0	0	0
II Mevalonic acid 500 mg/kg	2	0	1 (sac. moribund day 1 pp)[4]	1 (day 26 pc)[5]
III Fluvastatin 12 mg/kg, mevalonic acid 500 mg/kg	1	0	0	1 (day 18 pc)[4]
IV Fluvastatin 24 mg/kg, mevalonic acid 500 mg/kg	2	0	0	1 (day 19 pc)[3] 1 (elective sac. day 15 pc)[7]
V Fluvastatin 12 mg/kg	3	1[1]	1 (day 4 pp)[1] 1 (day 13 pp)[6]	0
VI Fluvastatin 24 mg/kg	8	1[2]	1 (day 2 pp)[2] 1 (day 5 pp)[2] 1 (day 6 pp)[2] 2 (sac. moribund day 10 pp)[2] 1 (day 12 pp)[2] 1 (day 14 pp)[2]	0

[1]Cause could not be determined.
[2]Heart lesion (vacuolar degeneration, and/or myocarditis probably related to death).
[3]Intubation accident, lesions seen by gross or microscopic examination.
[4]Intubation accident based on clinical observations and gross or microscopic examination.
[5]Possible intubation accident, although microscopic findings were not conclusive.
[6]Intubation accident based on clinical observations, although microscopic findings were not conclusive.
[7]Broken, malaligned incisors; animal in poor health.

pregnant animals, data from the nonpregnant animals were considered separately.

Based on results from previous studies, microscopic evaluations were limited to the hearts and stomachs from all animals. For spontaneously dying or animals sacrificed moribund, the tissue evaluation included gross lesions, lungs, trachea, thymus with mediastinum, and liver.

Statistical analysis

All statistical evaluations of data compared treatment groups with controls at $P \leq 0.05$ and $P \leq 0.01$ levels of significance, with a two-tailed analysis. Maternal body weight, duration of gestation, number of pups delivered, live pups per litter, pup weight, and implantation sites were evaluated by analysis of variance (ANOVA) followed by Dunnett's Test. Fisher's Exact Test was used to evaluate maternal fertility and reproductive performance, numbers of dead pups, and sex ratio, as well as neonatal necropsy examination data. Maternal terminal body weights were evaluated by a one-way ANOVA followed by Duncan's Multiple Range Test.

RESULTS

Maternal mortality and clinical observations

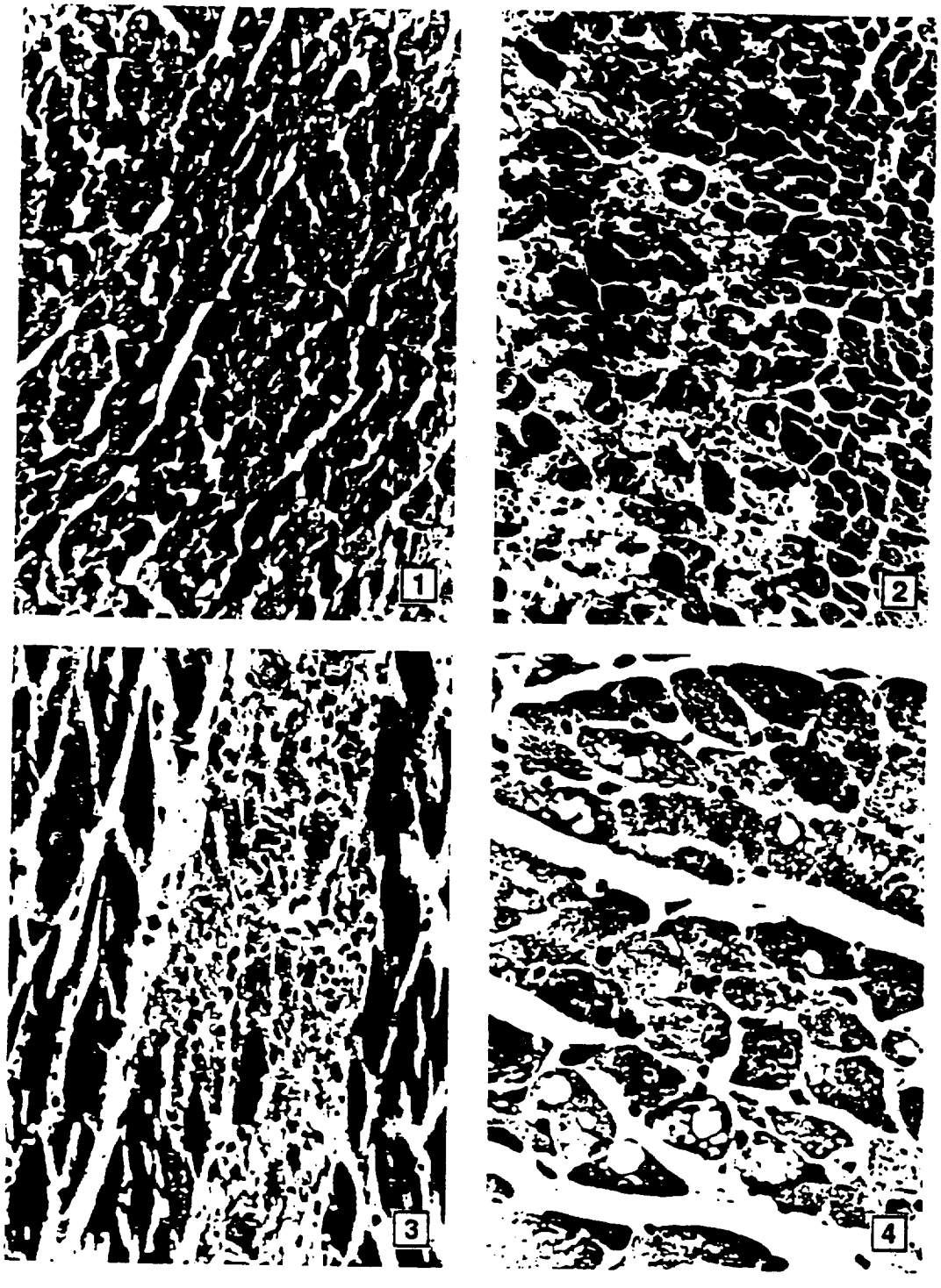
Drug-related maternal mortality occurred in animals receiving 12 and 24 mg/kg/day of fluvastatin where 2 out of 17 and 8 out of 17 pregnant animals, respectively, died or were sacrificed moribund (Table 2). One animal from each of these groups died on day 22 of gestation without completing delivery but had full-term fetuses in utero. Others died within the first 2 wk postpartum. The cause of death of the 8 animals administered fluvastatin at 24 mg/kg/day was related to the presence of cardiomyopathy characterized by hyaline, granular or vacuolar degeneration, and/or myocarditis (Fig. 2–5). The cause of death of the 2 rats administered fluvastatin at 12 mg/kg/day could not be determined.

Necropsy examination of the 2 animals that died on day 22 of gestation revealed dead, morphologically normal full-term fetuses in the uterus, indicating that they were probably alive up to the time of the dam's death. Furthermore, drug-related clinical observations were seen in the 24 mg/kg/day fluvastatin animal which included decreased locomotor activity (on days 20 and 21 pc), splayed (extended) hindlimbs, tremors, ataxia, labored breathing, salivation, lacrimation, and disorientation on day 21 pc. Similar signs were seen in the 12 mg/kg/day animal on day 22 pc prior to death.

Other animals from the 12 and/or 24 mg/kg/day fluvastatin groups also exhibited various drug-related clinical signs including ataxia, impairment/loss of righting reflex, decreased locomotor activity, hunched or flattened body position, splayed (extended) hind limbs, ptosis, tremors, disorientation, labored breathing, skin pallor, and dehydration.

Maternal body weight and food consumption

No treatment-related differences in maternal body weight gain were noted during gestation days 15–20, the initial period of administration of fluvastatin and/or mevalonic acid (Table 3). During the first 7 days after delivery, a transient, statistically significant ma-



Figs. 1–4.

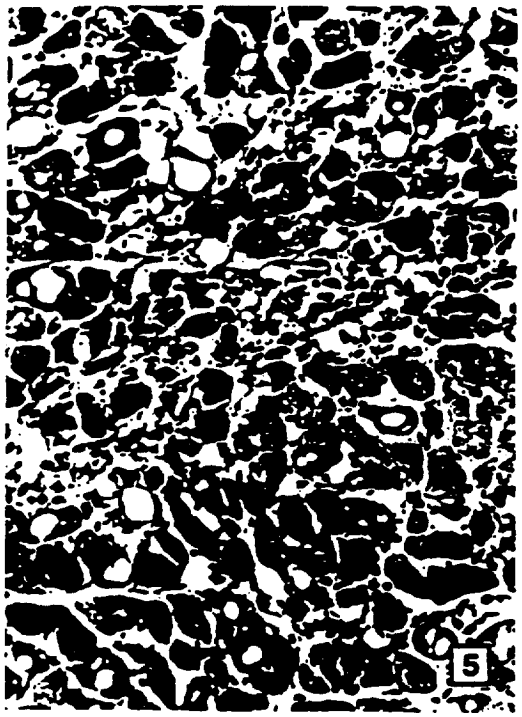


Fig. 5. 24 mg/kg: death on day 14 pp. In some high-dose animals dying in the later postpartum period, myocardial lesions encountered were often more complex, frequently characterized by areas of myofiber disintegration accompanied by inflammatory infiltrates and interstitial edema. The affected areas ranged from limited to widespread. Not seen in earlier myocardial lesions was the occurrence of conspicuous cytoplasmic vacuolization illustrated in this figure (× 400).

ternal body weight *loss* of 5% and 9% was apparent in the 12 and 24 mg/kg/day fluvastatin dose groups, respectively, compared to a 4% *gain* in controls. A 2% body weight *loss* occurred in animals receiving the 24 mg/kg/day dose level of fluvastatin supplemented with mevalonic acid. During the next 7 days (days 7–14 pp), a body weight gain was evident in all groups. However, considering the first 2 wk postpartum (days 0–14), an overall body weight loss was nevertheless apparent in the 2 fluvastatin groups (Table 3).

Subsequently, during days 14–21 pp, a slight maternal body weight loss was observed in all groups including controls. Maternal weight loss is not uncommon during this time period and is probably due to a greater nutritional demand on the dams resulting from increased lactation, combined with more ingestion of feed by the pups and therefore less by the dams. Although not statistically significant, a slight decrease in food consumption of approximately 11% was evident during the treatment period from days 15–20 of gestation in both groups administered 24 mg/kg/day of fluvastatin, with and without mevalonic acid (Table 3).

Fertility and reproductive performance

No remarkable variation in pregnancy rate occurred among the groups. Considering the mean length of the gestation period of those animals completing delivery, there was no treatment-related variation among groups (Table 4). A slightly lower gestation index in Group VI (fluvastain at 24 mg/kg/day) reflects the one animal which had no viable pups, 5 stillborn pups, and 7 dead pups of unknown viability status at birth.

Postnatal litter data

The mean number of liveborn pups was lower in both groups administered fluvastatin alone (Groups V and VI) when compared to concurrent controls (Table 5). An increased incidence of stillborn pups occurred in the 12 and 24 mg/kg/day fluvastatin Group V (7%) and Group VI (22%), compared to a range of 2–3% in controls and Groups II, III, and IV. There was no evidence to suggest any remarkable increase in in utero postimplantation embryo/fetal mortality (i.e., early or late resorptions) in any of the groups, either before or after initiation of fluvastatin and/or mevalonic acid administration (Table 5). Neonatal mortality of 15% and 56% was observed through weaning in both 12 and 24 mg/kg/day fluvastatin Groups V and VI, respectively (Table 5), with a statistically significant increase noted at 8–14 days postpartum in Group V and as early as 1–4 days postpartum in Group VI. Although the overall neonatal mortality in Group IV (24 mg/kg/day fluvastatin supplemented with mevalonic acid) was also statistically significant, it was minimal.

Drug-related lower birth weights as well as lower neonatal body weights during the lactation period were evident in both 24 mg/kg/day fluvastatin dose groups, with and without mevalonic acid supplementation.

The most prominent neonatal clinical observations occurred in Group VI (24 mg/kg/day of fluvastatin) where several litters, whose dams were adversely af-

Fig. 1. Control animal: typical appearance of nontreated control myocardial tissue (× 200).

Fig. 2. 24 mg/kg: death on day 21 pc before delivery. An example illustrating the significant but focal nature of myocardial hyaline degeneration, interstitial edema, and evidence of actual myofiber loss encountered in dying high-dose animals. Lesions of this type (left half of illustration) were detected only in animals dying during delivery or immediately postpartum. The myocardium in the right half of this illustration is unaffected (× 200).

Fig. 3. 24 mg/kg: death on day 22 pc before delivery. Discrete area of significant myocardial necrosis with mononuclear phagocytic infiltration. Affected myofibers have undergone granular degeneration. Adjacent myofibers are normal. Multiple foci of this type were seen elsewhere in the myocardium of this animal (× 200).

Fig. 4. 24 mg/kg: sacrificed moribund on day 10 pp. Conspicuous myofiber cytoplasmic vacuolization was frequently noted in animals dying later in the postpartum period. These cytoplasmic alterations were unaccompanied by inflammation or other evidence of myofiber degeneration. The distribution of affected fibers ranged from limited areas to widespread regions of myocardium. The vacuoles themselves were found to be devoid of fat or glycogen (× 400).

TABLE 3. Percent body weight change and relative food consumption compared to controls

Group	I Control	II Mevalonic Acid	III Fluvastatin + Mevalonic Acid	IV Fluvastatin + Mevalonic Acid	V Fluvastatin	VI Fluvastatin
	0 mg/kg/day	500 mg/kg b.i.d.	12 mg/kg/day + 500 mg/kg b.i.d.	24 mg/kg/day + 500 mg/kg b.i.d.	12 mg/kg/day	24 mg/kg/day
% body weight change:						
days 15–20 pc	18	18	18	16	16	16
0–7 pp	4	3	2	−2	−5*	−9*
0–14 pp	8	4	4	2	−1**	−3*
7–14 pp	4	1	2	4	4	1
14–21 pp	−5	−3	−4	−3	−3	−1
Relative food consumption: days 15–20 pc	100%	100%	96%	89%	93%	89%

*P ≤ 0.01.
**P ≤ 0.05.

TABLE 4. Fertility and reproductive performance

Group	I Control	II Mevalonic Acid	III Fluvastatin + Mevalonic Acid	IV Fluvastatin + Mevalonic Acid	V Fluvastatin	VI Fluvastatin
	0 mg/kg/day	500 mg/kg b.i.d.	12 mg/kg/day + 500 mg/kg b.i.d.	24 mg/kg/day + 500 mg/kg b.i.d.	12 mg/kg/day	24 mg/kg/day
No. of animals mated/group	20	20	20	20	20	20
# Pregnant	18	17	15	18	17	17
Pregnancy rate[1]	90%	85%	75%	90%	85%	85%
Gestation index[2]	100%	100%	100%	100%	100%	94%
No. of animals completing delivery	18	16[3]	15	18	16	16
Mean length gestation of animals completing delivery (days)	21.9	21.8	22.0	21.7	22.1	22.2
Range (days)	21–22	21–22	22	21–22	22–23	21–23

[1]Percent matings resulting in pregnancy; includes animals which died.
[2]Percent pregnancies resulting in litters with one or more viable offspring; does not include animals which died.
[3]Does not include one animal which delivered 4 stillborn, 4 dead, and 6 viable pups, but was sacrificed in a moribund condition on day 1 pp following a probable intubation accident on day 22 pc.

TABLE 5. Neonatal litter size and mortality

Group	Control	II	III	IV	V	VI
Mean no. of pups delivered	15.4	16.1	17.1	15.6	13.6	14.9
Mean no. of liveborn pups	15.1	15.9	16.7	15.2	12.6	11.1*
Mean no. of implantation sites	16.4	17.3	18.2	16.7	15.2	16.3
In utero post implantation loss	6.1%	6.9%	6.0%	6.6%	10.5%	8.6%
Overall neonatal mortality through weaning	3%	5%	6%	7%**	15%*	56%*

*P ≤ 0.01.
**P ≤ 0.05.

fected, had pups which appeared pale, thin, weak, and/or dehydrated. Necropsy examination of pups which were found dead during the lactation period, or were culled prior to weaning, showed that both groups administered fluvastatin alone (Group V and VI) had a higher incidence of pups without milk visible in the stomach.

Macroscopic and microscopic evaluation of spontaneously dying animals

No macroscopic evidence of treatment-related effects was noted other than the anticipated forestomach thickening present in 2 of 3 and 7 of 8 animals from the 12 and 24 mg/kg/day fluvastatin groups, respectively, which died or were sacrificed moribund.

TABLE 6. Time and incidence of treatment-related maternal mortality[1]

Study	Segment III			Segment III follow-up				Segment III mevalonate supplementation				
	Fluvastatin			Fluvastatin				MEV	Fluvastatin + MEV	Fluvastatin + MEV	Fluvastatin	
Dose (mg/kg)	2	12	24	2	6	12	24	500	12 + 500	24 + 500	12	24
Day 15–21 pc		2					2					
0 pp = 22 pc		2	4				4				1	1
23 pc		1	1									
1–4 pp			1				2				1	1
5–15 pp		1	6			1	3					6

[1]To facilitate simultaneous presentation of data, the control groups (all negative) were omitted.

Microscopic findings consisted of a variety of myocardial lesions in all dying Group VI (fluvastatin at 24 mg/kg/day) animals. The cardiomyopathy was characterized by focal myocarditis and lesions of myofiber hyaline, granular and vacuolar degeneration (Table 2). These findings were considered treatment-related and similar to those seen in a previous study in pregnant or lactating animals dying during or after parturition.

Two types of myocardial lesions were detected microscopically. In animals dying at and within a few days after parturition, the lesions were characterized by hyaline degeneration of isolated groups of myofibers accompanied by slight interstitial edema and hemorrhage (Fig. 2 and 3). The lesions typical of animals dying 3–4 days after parturition or later were characterized by widespread cytoplasmic vacuolar changes in the myofibers, i.e., the occurrence of large and small clearly defined round empty vacuoles (Fig. 4 and 5). Occasionally these were accompanied by a sarcolemmal response and inflammatory cell infiltrates, which suggested a myofiber proliferative or reparative response to the induction of the myocardial injury.

Cardiomyopathy was not present in the spontaneously dying animals which had been given a lower dose (12 mg/kg/day) of fluvastatin alone. All animals dying in the 24 mg/kg/day fluvastatin group had cardiac lesions. These lesions were considered related to the cause of death in these animals.

Terminal sacrifice animals

The only treatment-related effect noted occurred in all the groups which received fluvastatin. Most of the animals in Group III (14 out of 19), Group IV (17 out of 18), Group V (14 out of 17), and Group VI (11 out of 12) had varying degrees of forestomach thickening. No evidence of this effect was detected in Group II (mevalonic acid alone) or the controls. In Groups III and IV, supplementation with mevalonate did not influence the incidence of fluvastatin-induced forestomach thickening.

Microscopically, the forestomach thickening was characterized by epithelial hyperplasia/hyperkeratosis in all groups which received fluvastatin, including those receiving the mevalonic acid supplementation. No evidence of ulceration accompanied these hyperplastic lesions. The hyperplasia ranged from mild to moderate in degree. In the absence of specific quantitative measurements, there appeared to be correlation, with greater degrees of hyperplasia seen at the higher doses of fluvastatin in Groups IV and VI (24 mg/kg/day) compared to the response seen in Groups III and V (12 mg/kg/day). There did appear to be a lesser degree of hyperplasia/hyperkeratosis in the animals administered mevalonate and 12 mg/kg/day fluvastatin (Group III) compared to the low dose of fluvastatin alone (Group V).

Histological evidence of fluvastatin-induced myocardial fiber injury and/or inflammation was not present in the mevalonate-supplemented Groups III and IV. Likewise, in the surviving Group V and VI (fluvastatin at 12 and 24 mg/kg/day) animals, myocardial lesions were not detected.

DISCUSSION

The maternal toxic effects induced by fluvastatin reported herein had been encountered in two previous studies at similar dose levels, and consisted of maternal mortality at delivery and during the postpartum period (Table 6). Microscopic evidence of associated myocardial lesions was detected in the follow-up study. An increase in stillborn pups was observed with no evidence of earlier in utero fetal mortality, suggesting a toxic effect on the dams immediately prior to, or during, parturition. The absorption and disposition of fluvastatin have been studied in nonpregnant rats (Tse et al., '90) and in pregnant and lactating rats, and in suckling pups (A. Schweitzer, unpublished data). In all cases fluvastatin was rapidly eliminated with little or no accumulation in the tissues. Limited placental transfer resulted in very low levels in embryos or fetuses. Fluvastatin was detected in maternal milk and subsequently at measurable levels in suckling pups. However, the neonatal mortality observed soon after parturition, as well as during the lactation period, may be a reflection of adverse effects relative to deficient maternal lactation and lack of litter care as influenced by maternal toxicity.

The results indicate that fluvastatin-induced maternal and neonatal mortality could be completely blocked

and/or ameliorated by coadministration of mevalonic acid. Adverse effects on maternal body weight were nonexistent with mevalonic acid supplemented fluvastatin at 12 mg/kg/day and markedly improved at 24 mg/kg/day, although food consumption remained slightly decreased at 24 mg/kg/day despite supplementation with mevalonic acid. The decreased neonatal body weight gain during lactation at 24 mg/kg/day may be related to the reduced maternal food intake noted at this dose level.

While mevalonate supplementation prevented mortality and the development of heart lesions in Groups III and IV (fluvastatin at 12 and 24 mg/kg/day), it did not reduce the direct effect of fluvastatin on the gastric forestomach squamous epithelium, although the degree did appear somewhat lower in Group III. This is considered further evidence that hyperplasia/hyperkeratosis is the result of direct or contact irritation by fluvastatin on the squamous forestomach epithelium.

The fluvastatin-induced early cardiac lesions characterized by hyaline degeneration (before or at parturition) resembled those induced by isoproterenol (pregnant rats are uniquely sensitive)[1]. The postnatal lesions (vacuolar sarcoplasmic changes and myocarditis) appeared to be the result of sarcoplasmic fluid accumulation possibly related to alteration of cellular membrane structures.[1] The relationship or progression between pre- and postpartum lesions, if any, is not clear. Likewise, it is not known at what point in their development either would be reversible.

Several factors existed which probably contributed to the development of myocardial damage and/or death of the fluvastatin-treated animals. In addition to the concurrent stresses of pregnancy, parturition, and lactation, the exaggerated pharmacologic activity of fluvastatin, i.e., inhibition of mevalonate and cholesterol biosynthesis, was a significant factor. HMG-CoA reductase and its immediate product mevalonic acid play a key role in cholesterol biosynthesis and are essential for the synthesis of steroid hormones, cell membranes, cell growth, and normal cellular metabolism (Brown and Goldstein, '80; Alberts, '88; MacDonald et al., '88). It would not be unexpected, therefore, that inhibition of this critical enzyme would influence or disturb the dynamic events occurring in late gestation and at parturition. The data from Groups III and IV clearly indicate that mevalonate supplementation prevents mortality and the development of heart lesions. It is not clear from these studies exactly what the effect is in the myocardial tissues which allows development of the heart lesions, and why the restored or maintained levels of mevalonate suppress or block the development of these lesions.

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The mechanism underlying the hypolipemic effect of perfluorooctanoic acid (PFOA), perfluorooctane sulphonic acid (PFOSA) and clofibric acid

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The influence of the peroxisomal proliferators perfluorooctanoic acid (PFOA), perfluorooctane sulphonic acid (PFOSA) and clofibric acid on lipid metabolism in rats was studied. Dietary treatment of male Wistar rats with these three compounds resulted in rapid and pronounced reduction in both cholesterol and triacylglycerols in serum. The concentration of liver triacylglycerols was increased by about 300% by PFOSA. Free cholesterol was increased by both perfluoro compounds. Cholesteryl ester was reduced to 50% by PFOSA as well by clofibrate. In hepatocytes from fed rats, all the compounds resulted in reduced cholesterol synthesis from acetate, pyruvate and hydroxymethyl glutarate, but there was no reduction of synthesis from mevalonic acid. The oxidation of palmitate was also increased in all groups. The perfluoro compounds, but not clofibrate, caused some reduction in fatty acid synthesis. The activity of liver HMG-CoA reductase was reduced to 50% or less in all treatment groups and all three compounds led to lower activity of acyl-CoA:cholesterol acyltransferase (ACAT). Changes in other enzymes related to lipid metabolism were inconsistent. The present data suggest that the hypolipemic effect of these compounds may, at least partly, be mediated via a common mechanism; impaired production of lipoprotein particles due to reduced synthesis and esterification of cholesterol together with enhanced oxidation of fatty acids in the liver.

Introduction

Many hypolipemic drugs cause proliferation of peroxisomes and increase the activity of the peroxisomal β-oxidation in rats [1–3]. Chemically, these drugs constitute a heterogeneous group including clofibrate, tibric acid, niadenate and long-chain acylthioacetic acids, tiadenol, long-chain thia acids and MEDICA 16 [1–6]. It has been suggested that the increase in the fatty acyl-CoA oxidizing system contributes to the hypolipemic effect of these drugs [7,8]. The dominating mechanism underlying reduction of serum triacylglycerols and cholesterol by these drugs is, however, uncertain. Other mechanisms which may be important for the hypolipemic effect include; reduced hepatic synthesis of fatty acids and cholesterol [9–12]; reduced triacylglycerol release by the liver [13,14]; increased rate of VLDL degradation [6]; increased uptake or reduced release of fatty acids by adipose tissue [15,16] and increased excretion of cholesterol into bile and feces [17].

Ikeda et al. [18,19] observed that perfluorooctanoic acid (PFOA) and perfluorooctane sulphonic acid (PFOSA) efficiently induced the peroxisomal β-oxidation in rats that were fed these compounds (0.02% in the diet). Just et al. [20] reported that the perfluorocarboxylic acids alter hepatic lipid metabolism and reduce serum lipid levels showing that these compounds also belong to the group of peroxisomal proliferators with hypolipemic effect. The perfluorinated compounds are particularly interesting since they obviously are not subject to ordinary metabolic modifications. The effects of these compounds must, therefore, be due to effects of the compounds per se.

Peroxisomal inducers may bring about the hypolipemic effect by affecting different steps in lipid metabolism. However, it seems likely that some important steps in lipid metabolism are common targets for these compounds. The unphysiological character of the perfluoro compounds makes it possible that their effect

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Abbreviations: PFOA, perfluorooctanoic acid; PFOSA, perfluorooctane sulphonic acid; ACAT, acyl-CoA: cholesterol acyltransferase; HMG, hydroxymethyl glutaric acid; TTA, tetra-decylthioacetic acid; VLDL, very-low-density lipoproteins.

on lipid metabolism follows a pattern that would make it easier to understand possible mechanism for the lipid reduction effect of peroxisomal proliferators.

In the search for a possible common mechanism underlying the hypolipemic effect of peroxisomal inducers, we have compared hepatic fatty acid metabolism, cholesterol synthesis and the activities of enzymes related to these metabolic processes in the liver of rats fed perfluorooctane sulphonic acid (PFOSA), perfluorooctanoic acid (PFOA) and clofibrate.

Methods and Materials

Animals

Male Wistar rats were used. They were divided into five groups. One group was allowed normal food ad libitum (control rats). Three other groups were given food which contained 0.3% clofibric acid, 0.02% perfluorooctanoic acid or 0.02% perfluorooctane sulphonic acid, respectively. The diets were prepared by soaking standard food (in pellet form) in diethylether in which the compounds had been dissolved. The fifth group was restricted in food intake to that consumed by the PFOSA group. The stock diet was used for these paired feeding experiments. The average body weight when the experiments started was 269 g and the average daily food consumption per rat in the different groups were; control: 23.7 g, clofibrate: 23.1 g, PFOA: 22.7 g, PFOSA: 20.0 g.

Materials

[2-^{14}C]Mevalonic acid, [1-^{14}C]pyruvic acid and [2-^{14}C]pyruvic acid were from New England Nuclear. [1-^{14}C]acetic acid was obtained from Amersham (UK). Clofibrate was from Fluka (Buchs, Switzerland). Perfluorooctanoic acid was purchased from Aldrich-Chemie (Steinheim, Germany) and Perfluoro-octane sulphonic acid was from Fluorochem (Old Glossop, UK). Other chemicals were from Sigma (St. Louis, MO, USA).

Preparation of hepatocytes

The effects of dietary treatment were studied in hepatocytes isolated from rats fed the diets for 1 week and for the study of direct effects of the compounds, hepatocytes were isolated from rats fed the standard diet. Isolation of hepatocytes was performed by perfusion with collagenase, according to Berry and Friend [21], with the modifications described by Seglen [22].

Oxidation of palmitate and conversion of labelled substrate into lipids

Fatty acid oxidation was measured according to Christiansen et al. [23] with 0.5 mM palmitate as substrate. Fatty acids and cholesterol synthesized from radioactive precursors were extracted from the cell suspension after 90 min incubation. The reaction was stopped by the addition of 5% saturated KOH in ethanol and the mixture was heated at 90°C for 1 h. The nonsaponifiable lipids were extracted with petroleum ether. The suspension was then acidified with HCl. The extracts were evaporated to dryness and the radioactive lipid residue was dissolved in 100 μl hexane. The lipid extracts were chromatographed with hexane/diethyl ether/acetic acid (80:20:1) on silica gel thin-layer plates. The spots corresponding to cholesterol and fatty acids were identified by standards and were then isolated for measurement of radioactivity.

Enzyme assays and measurements of DNA, protein and lipids

Pyruvate dehydrogenase was estimated by measuring the $^{14}CO_2$ liberated when hepatocytes were incubated with 5 mM [1-^{14}C]pyruvate for 30 min at 37°C. Acetate thiokinase was measured as described by Jones and Lipman [24]. Liver microsomes were prepared according to Easom and Zammit [25], and HMG-CoA reductase was measured according to Drevon et al. [26]. CDPcholine : 1,2-diacylglycerol cholinephosphotransferase, EC 2.7.8.2 and lysolecithin acyltransferase

TABLE I

Body and liver weights and liver lipid content in rats fed different diets for 7 days

Clofibrate was given as 0.3% (w/w) and PFOA and PFOSA as 0.02% in the diet. Values are given as means ± S.E. There were four observations in each group. Fisher's *P*-values are given; * $P < 0.05$; ** $P < 0.01$ vs. control group.

	Control	Clofibrate	PFOA	PFOSA	Stock diet limited fed
Body weight (g)	305 ± 2	298 ± 3	288 ± 6 *	275 ± 6 **	282 ± 7 **
Liver weight (g)	11.3 ± 0.4	16.1 ± 0.8 **	18.8 ± 0.7 **	15.9 ± 0.7 **	
Liver triacylglycerols (μmol/g liver)	4.1 ± 0.5	4.0 ± 0.7	3.9 ± 0.3	13.8 ± 18 **	4.1 ± 0.6
Liver non-esterified cholesterol (μmol/g liver)	4.4 ± 0.4	4.5 ± 0.3	6.5 ± 0.4 *	7.6 ± 0.3 **	
Liver cholesterol ester (μmol/g liver)	0.58 ± 0.08	0.24 ± 0.02 **		0.27 ± 0.03 *	

(EC 2.3.1.23) was measured as described by Parthasarathy et al. [27]. Acyl-CoA : cholesterol acyltransferase (ACAT) was measured according to Rustan et al. [28] and the synthesis of phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine was measured as described by Vance [29]. Other enzymes were measured as described earlier [30]. DNA was measured by the method of Labarca and Paigen [31] and protein was estimated by the biuret method or by the method of Lowry et al. [32].

Liver lipids were extracted with chloroform/methanol (2 : 1, v/v). Triacylglycerols was determined directly on the dried extract with a kit method (Nyco, Oslo, Norway). Free and esterified cholesterol was determined by Nycotest kit method for cholesterol (Nyco) after separation of the extract on thin-layer chromatography. Serum cholesterol and triacylglycerols was measured directly by the kit methods.

Results

Table I shows that 0.02% PFOA or PFOSA in the diet resulted in a lower body weight after 7 days of feeding as compared with the control group. The clofibrate diet (0.3%) did not affect the rat weight. The group with restricted food intake to that of the PFOSA group had about the same weight as the PFOSA group. All the compounds resulted in a 40–60% increased liver weight. Similar effect on the liver weight has been observed earlier in rats fed clofibrate [9,10,33].

Changes in serum and liver lipids by clofibrate, PFOA and PFOSA

Fig. 1A shows that all three diets significantly reduced serum cholesterol. In all treatment groups, cholesterol was significantly reduced (to 50–70% of control) after 24 h. Dietary treatment for 2 weeks resulted in further cholesterol reductions by 70% or more. In agreement with other observations, fasting for 2 days did not bring about significant cholesterol changes.

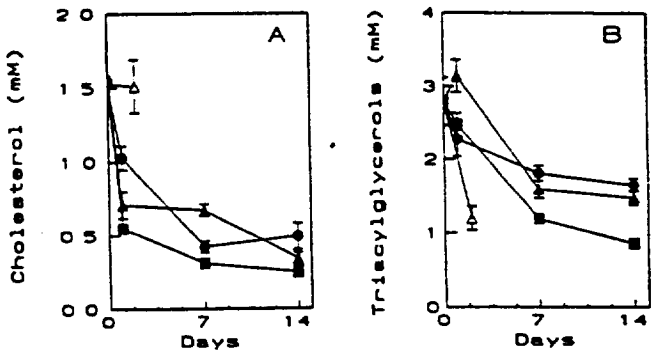

Fig. 1. Effect of clofibrate, PFOA and PFOSA on the serum lipids. (A) Cholesterol; (B) triacylglycerols; △, fasted; ●, 0.3% clofibrate; ▲, 0.02% PFOA; ■, 0.02% PFOSA. The data represent mean ± S.E. of four observations.

Fig. 1B shows the effects of clofibrate, PFOA and PFOSA on triacylglycerols in the rat serum. None of the compounds resulted in significant changes after 1 day of treatment. After 7 days, clofibrate and PFOA resulted in reduction to about 60% of control value and no further reduction was obtained with further treatment. PFOSA reduced triacylglycerols to about 50% and 30% of control value after 1 and 2 weeks of treatment. The significant reduction obtained by fasting for 2 days was as expected. In the rats fed stock diet but restricted to that consumed by the PFOSA group, the serum triacylglycerols was 2.43 ± 0.13 mM after 1 week of treatment (not shown) which is not significantly lower than observed in the control group (fed stock diet ad libitum).

Table I shows that PFOSA increased the liver triacylglycerols content to more than 3-times the control value. This effect of PFOSA on triacylglycerols was clearly in contrast to the effect of clofibrate and PFOA which did not affect the content of triacylglycerols and clearly indicates that PFOSA inhibits the excretion of triacylglycerols from the liver. Both PFOA and in particular PFOSA increased the liver content of non-esterified cholesterol. In contrast to this, a significant

TABLE II

Conversion of labelled substrates into ^{14}C-labelled cholesterol by hepatocytes of rats fed different diets for 7 days

Clofibrate was given as 0.3% (w/w) and PFOA and PFOSA as 0.02% in the diet. The concentration of [2-^{14}C]mevalonate and [3-^{14}C]hydroxymethylglutarate was 0.5 mM. Incubations with [1-^{14}C]acetate and [2-^{14}C]pyruvate were conducted with 5 mM of the labelled substrates and included also unlabelled glucose (10 mM). Values are means ± S.E. There were four observations in each group. Fisher's P values are given * $P < 0.05$; ** $P < 0.01$.

Substrate	Formation of ^{14}C-labelled cholesterol (nmol substrate carbon · mg DNA^{-1} · h^{-1})			
	control	clofibrate	PFOA	PFOSA
[1-^{14}C]Acetate	147.6 ± 21.4	59 ± 13 **	37.6 ± 16.5 *	21.2 ± 7.4 **
[2-^{14}C]Pyruvate	78.2 ± 14.6	46 ± 9.5 **	35.2 ± 9.7 *	26.5 ± 5.4 **
[3-^{14}C]Hydroxy-methylglutarate	17.9 ± 3.3	3.6 ± 0.9 **	3.6 ± 1.3 **	5.0 ± 1.1 **
[2-^{14}C]Mevalonate	2280 ± 150	2160 ± 280	1730 ± 240	2030 ± 310

TABLE III

Conversion of labelled substrates into ^{14}C-labelled fatty acids and oxidation of [U-^{14}C]palmitate by hepatocytes of rats fed different diets for 7 days

The concentrations of compounds in the diet and the concentrations of [1-^{14}C]acetate and [2-^{14}C]pyruvate were as described in Table II. The concentration of [U-^{14}C]palmitate was 0.5 mM. Values are means ± S.E. There were 4 observations in each group. Fisher's P values are given: * $P < 0.05$; ** $P < 0.01$ vs. control group.

Substrate	^{14}C-Labelled fatty acid formation (nmol substrate carbon·mgDNA^{-1}·h^{-1})			
	control	clofibrate	PFOA	PFOSA
[1-^{14}C]Acetate	252 ± 28	180 ± 28	90 ± 54 *	104 ± 28 **
[2-^{14}C]Pyruvate	221 ± 21	322 ± 23 *	182 ± 55	128 ± 21 *
	[U-^{14}C]Palmitate oxidation (nmol substrate oxidized·mgDNA^{-1}·h^{-1})			
	control	clofibrate	PFOA	PFOSA
	502 ± 50	726 ± 36 *	564 ± 63	861 ± 102 *

TABLE IV

Conversion of [1-^{14}C]acetate into ^{14}C-labelled fatty acids and cholesterol by hepatocytes in the presence of hypolipemic compounds

The concentration of [1-^{14}C]acetate was 5 mM and the incubations also included 10 mM glucose. Each incubation flask contained 4 mg cell protein and 1.5% albumin in 3 ml Krebs-Henseleit bicarbonate buffer. The concentration of hypolipemic compounds was 1 mM. Values are means ± S.E. There were four observations in each group. Fisher's P values are given ** $P < 0.01$ vs. control group.

Addition	Formation of ^{14}C-labelled cholesterol	Formation of ^{14}C-labelled fatty acid
	(nmol substrate carbon mg DNA^{-1}·h^{-1})	
None	114 ± 8	313 ± 14
TTA	1.4 ± 0.07 **	7.5 ± 1.3 **
Clofibric acid	57 ± 0.7 **	243 ± 8 **
PFOA	63 ± 11 **	611 ± 20 **
PFOSA	65 ± 7 **	1908 ± 29 **

reduction (to approx. 50%) in esterified cholesterol in the treated rats (PFOA-treated rats not measured) was found.

Conversion of labelled substrates into cholesterol by hepatocytes of rats fed different diets

Table II shows the incorporation of labelled carbon atoms from differently labelled acetate, pyruvate, mevalonate and hydroxymethylglutaric acid (HMG). The major point emerging from this table is that the rate of cholesterol synthesis was significantly reduced from all substrates which are proximal to the HMG-CoA dehydrogenase step in all treatment groups. In contrast, cholesterol synthesis from mevalonate was not reduced in any of the groups.

Table III shows the synthesis of fatty acids from pyruvate and acetate. Both PFOA and PFOSA treatment reduced lipid synthesis (not significant from pyruvate in the PFOA group). No reduction in fatty acid synthesis was found in hepatocytes from clofibrate-treated animals. The table further shows that the peroxisomal inducers, as expected, resulted in an increased rate of palmitate oxidation (although PFOA did not result in a significant increase). The experiments do not exclude the possibility that intracellular content of the tested compounds might have a direct (reversible) effect on fatty acid and cholesterol synthesis in vivo. Such effects could have escaped our detection since the compounds might have been washed out during the cell isolation procedure. To test possible direct inhibitory effects of these drugs, the synthesis of fatty acids and cholesterol in hepatocytes with additions of the compounds to the incubation medium were performed (Table IV). The table shows that 1 mM of clofibric acid, PFOA and FPOSA inhibited the choles-

TABLE V

Activity of enzymes related to the synthesis of cholesterol and fatty acids in liver of rats fed different diets for 7 days

The concentrations of compounds in the diet were as described in Table I. Values are given as means ± S.E. There were four observations in each group. * $P < 0.05$; ** $P < 0.05$; ** $P < 0.01$ vs. control group.

Enzyme	Activity (μmol·(mg DNA^{-1}·min^{-1})			
	control	clofibrate	PFOA	PFOSA
Pyruvat dehydrogenase	0.59 ± 0.05	0.58 ± 0.04	0.42 ± 0.08	0.36 ± 0.04 *
Citrate synthase	0.97 ± 0.12	0.94 ± 0.06	0.80 ± 0.05	1.10 ± 29
ATP-citrate lyase	1.54 ± 0.34	0.78 ± 0.38	0.75 ± 17 *	0.24 ± 0.06 **
Acetate thiokinase	8.7 ± 0.64	5.7 ± 0.85	3.5 ± 0.5 **	5.7 ± 0.28 **
Malate dehydrogenase (NADP) (decarboxylating)	0.36 ± 0.05	0.83 ± 0.05 **	1.34 ± 0.05 **	0.21 ± 0.01
Malate dehydrogenase	27.1 ± 3.2	18.4 ± 2.4 *	20.7 ± 2.6	19.6 ± 3.0
Glucose-6-phosphate dehydrogenase	0.50 ± 0.10	0.17 ± 0.03 *	0.23 ± 0.04 *	0.11 ± 0.01 **
Isocitrate dehydrogenase (NADP)	2.44 ± 0.35	2.71 ± 0.56	2.73 ± 0.19	3.23 ± 0.13
	(nmol·μg protein^{-1}·min^{-1})			
HMG-CoA reductase	0.31 ± 0.03	0.16 ± 0.04 *	0.15 ± 0.05 *	0.11 ± 0.01 **

terol synthesis to the same extent (approx. 50%). Surprisingly, tetradecylthioacetic acid (TTA), another inducer of peroxisomal β-oxidation with hypolipemic effect in rats [3,4], almost completely inhibited cholesterol synthesis. The pronounced inhibitory effect of TTA on the fatty acid synthesis observed by Skrede et al. [34] was also confirmed. Clofibric acid reduced the fatty acid production by about 20%. However, a direct inhibitory effect on the fatty acid synthesis is not a property shared by all the tested compounds. Both perfluorinated compounds unexpectedly stimulated the rate of fatty acid synthesis strongly. It is unlikely that this was due to an inhibition of Krebs cycle with a concomitant increase in lipogenic precursors, since these compounds inhibited the cholesterol synthesis. It seems more likely that the compounds stimulate a rate-limiting enzyme, e.g., acetyl-CoA carboxylase. At lower concentrations (0.5 and 0.1 mM), there were very small inhibitory effects of clofibric acid, PFOA and PFOSA (data not shown).

Enzymes related to the synthesis of cholesterol and fatty acids from pyruvate and acetate

Table V shows that pyruvate dehydrogenase, citrate synthase and acetate thiokinase were only slightly affected by treatment with the three compounds. ATP-citrate lyase activity was reduced to about 15% of control values by PFOSA. PFOA reduced the activity of this enzyme significantly to 50%. The effect of the compounds on three NADPH-generating enzymes shows a remarkable pattern. All three compounds significantly reduced the activity of glucose-6-phosphate dehydrogenase. PFOSA reduced the activity to 20% of control values. In contrast, isocitrate dehydrogenase was unaffected by all three compounds. The activity of malic enzyme was increased 2- and 3.5-fold by clofibrate and PFOA, respectively. Malate dehydrogenase, which is not specifically involved in lipid synthesis, was virtually unchanged by any of the compounds. The activity of HMG-CoA reductase, the rate-limiting and regulated step in cholesterol synthesis, was reduced to 50% or less in all three treatment groups.

Enzymes related to the synthesis of cholesteryl ester and phospholipids in rats fed different diets

Table VI shows that acyl-CoA: cholesterol acyltransferase (ACAT), was significantly downregulated by all three compounds. PFOSA, which had the strongest effect, reduced the activity to one third of control. The downregulation of this enzyme is in keeping with the reduction of liver cholesterol ester (Table I). The table further shows that the activities of two enzymes important in the phopholipid turnover, acyl-CoA : 1-acylglycero-3-phosphocholine acyltransferase and CDP-choline : 1.2-diacylglycerol cholinephosphotransferase, were not significantly altered in the rats fed any of the hypolipemic drugs. The activity of phosphatidylserine synthase, phosphatidylethanomaline synthase and phosphatidylcholine synthase were also unaffected by any of the dietary regimes utilized (not shown).

Activities of enzymes related to phospholipid synthesis in the presence of hypolipemic drugs

Table VII shows that at 0.5 mM clofibric acid had little effect on activities of the enzymes listed in the table. The five enzymes were all inhibited by both perfluorinated. compounds. PFOA had a particular inhibitory effect on phosphatidylserine synthase activity which was reduced to 18% of normal with 0.5 mM PFOA. PFOSA had strongest inhibitory effect on the activity of CDP-choline : 1,2-diacylglycerol cholinephosphotransferase, phosphatidylserine synthase and phosphatidylethanolamine synthase, which were reduced to 14%, 13% and 28% of control, respectively with 0.5 mM PFOSA. At lower concentration (0.1 mM) the inhibitory effects of the perfluorinated compounds were very moderate (data not shown). Clofibric acid had no significant effect at 1 mM concentration.

TABLE VI

Activity of enzymes related to synthesis of cholesteryl esters and phospholipids in livers of rats fed hypolipemic drugs for 7 days

The concentrations in the diet were as described in Table I. Values are given as means ± S.E. There were four observations in each group. ** $P < 0.01$ vs. control group.

Enzyme	Control	Clofibrate	PFOA	PFOSA
Acyl-CoA Cholesterol acyltransferase (ACAT) (nmol/mg protein per min)	660 ± 51	427 ± 18 **	323 ± 39 **	237 ± 41 **
Acyl-CoA : 1-acylglycero-3-phosphocholine acyltransferase (nmol/mg protein per min)	14.0 ± 1.3	12.4 ± 1.0	12.7 ± 1.3	11.8 ± 1.8
CDP-choline : 1,2-diacylglycerol cholinephosphotransferase (nmol/mg protein per min)	10.4 ± 1.3	9.1 ± 1.2	7.6 ± 0.7	8.6 ± 1.0

TABLE VII

Activities of enzymes related to phospholipid synthesis in the presence of hypolipemic drugs

Concentrated solutions (10 mM) of clofibric acid, PFOA and PFOSA in DMSO were diluted to 0.5 mM final concentration in assay system (The controls were added the same amount of pure DMSO.) The effect of the drugs on each of the enzymes were tested four times and in each of the experiments, the activities were calculated as per cent of the control value. Values are given as means ± S.E.

	Control	Clofibric acid	PFOA	PFOSA
Acyl-CoA lysophosphatidyl transferase	100	90 ± 6	56 ± 7	61 ± 5
CDP-choline: 1,2-diacylglycerol cholinephosphotransferase	100	95 ± 5	71 ± 2	14 ± 0.6
Phosphatidylserine synthase	100	101 ± 9	18 ± 4	13 ± 1.4
Phosphatidylethanolamine synthase	100	101 ± 3	47 ± 5	28 ± 2
Phosphatidylcholine synthase	100	89 ± 3	47 ± 5	48 ± 3

The effect of the compounds on the activity of ACAT was tested only at 100 μM and with this concentration the ACAT activity was unaffected by the compounds (not shown).

Discussion

Reduction of the steady-state levels of serum lipids may be visualized as the result of downregulation of lipid synthesis or increased clearance from plasma. The aim of this study was to evaluate the effects of three different peroxisomal proliferators on a series of enzymes involved in hepatic lipid synthesis. The most important observations with all three compounds was downregulation of HMG-CoA reductase, the rate-limiting enzyme of cholesterol synthesis and of cholesterol esterification enzyme (ACAT). The agreement between the observed alterations in the enzyme activities and production of cholesterol and lipids in intact liver cells provides some support for the assumption that such enzyme measurements do reflect real changes in metabolic activity in the intact organ and shed light on the mechanism of the hypolipemic effects of the agents investigated in this study. These compounds do not show a correlated effect on the lipogenic and cholesterogenic pathways in the in vivo experiments. The in vitro experiments indicate that there are direct, presumably reversible, effects on these pathways. These effects are different than those observed after dietary manipulation. The latter probably represent changes in enzyme concentrations since, at least, the changes in the cholesterogenic pathway are correlated with the changes in HMG-CoA reductase.

The perfluorinated compounds utilized in this study are strong local irritants. The observed actions should not be regarded as mere unspecific toxic effects since, in addition to reduction of the activity of some enzyme systems, these compounds increase the activity of other enzymes, e.g., enzymes of the peroxisomal fatty acid β-oxidation system [18,19]. However, local irritation may explain the reduction of food intake and slower weight increase observed after feeding perfluorinated compounds. However, this probably contributes little to the reduction in serum triacylglycerols, since no significant reduction was observed in rats with restricted food intake. Increased liver weight correlates well with earlier studies on peroxisomal proliferators [35,36].

Cholesterol and fatty acid synthesis

The reduction of cholesterol synthesis from different labelled substrates fits well with the reduced activity of HMG-CoA reductase observed in this study. Three substrates, proximal to the reductase step, were incorporated into cholesterol at a reduced rate whereas no reduction from mevalonate was observed in any of the treatment groups. Lowering of HMG-CoA reductase levels by clofibrate is in accord with other observations [9,37,38]. Even though the three compounds tested in this study reduce the HMG-CoA reductase activity, this effect may not be a characteristic of all peroxisomal inducers. MEDICA 16, another compound in this group, does not act via an effect on this reductase [12,39], but rather inhibits the synthesis of cholesterol at a step distal to HMG-CoA reductase. Our observations with clofibrate are at variance with the data obtained by Azarnoff et al. [10] who found that cholesterol synthesis from mevalonic acid was reduced in livers of rats fed clofibrate.

In contrast to the inhibitory effect of MEDICA 16 on ATP-citrate lyase [39], clofibric acid, PFOA and PFOSA had essentially no direct effect on ATP-citrate lyase at 1 mM (data not shown), but the drugs downregulated the enzyme after dietary administration. This effect may contribute to reduced synthesis of cholesterol in vivo, since the enzyme is important in the main pathyway for cholesterol precursor synthesis. It is interesting that all three compounds reduced one of the NADPH generating enzymes (glucose-6-phosphate dehydrogenase), while isocitrate dehydrogenase (NADP) was unchanged. Reduced capacity for NADPH generation can therefore hardly contribute to reduction in serum lipid levels.

The synthesis of fatty acids from acetate or pyruvate was not reduced in hepatocytes from rats fed clofibrate (Table III), but was significantly reduced in the hepatocytes from rats fed PFOSA. The reduced rate of fatty acid synthesis in the PFOA and PFOSA groups is probably not related to reduction in serum triacylglycerols. In rats fed PFOSA, there was accumulation of liver triacylglycerols. The lack of reduction of fatty acid synthesis by clofibrate suggests that other pro-

cesses in lipid metabolism must be more essential for the hypolipemia. Tomarelli et al. [40] found increased synthesis of lipids from acetate in rats fed clofibric acid and also in rats fed the very potent hypolipemic compound WY-14,643.

Synthesis of cholesteryl ester and phospholipids

Liver exports lipids to other organs mainly as VLDL particles. In addition to apolipoproteins, triacylglycerols and free cholesterol, these particles consist mainly of esterified cholesterol and phospholipids. Hence, reduced hepatic synthesis of these components might lead to reduced transport of lipids from the liver.

The hepatic content of cholesteryl ester was reduced both in clofibrate and PFOSA fed rats (PFOA fed rats were not tested) even where free cholesterol was not reduced (Table I). Similar effect was obtained by Avignan et al. [41] with the hypolipemic drug MER-29. Reduced cholesteryl ester production is most likely a result of downregulation of the ACAT activity which was observed in all treatment groups. A direct inhibitory effect on the enzyme by the compounds is probably of less importance since 100 μM of the compounds did not affect the enzyme activity (higher concentrations were not tested). Vance [29] argued that synthesis of phospholipids may be limiting for lipoprotein synthesis. In this study, we have not observed any downregulation of enzymes involved in the synthesis or metabolism of phospholipids. We have observed only small direct effects of clofibric acid on these enzymes. It seems likely, however, that reduced phospholipid synthesis plays a role in the lipid-reducing effect of the perfluorinated compounds, since these had direct inhibitory effect on several important enzymes. Parthasarathy et al. [27] suggested from their studies that inhibition of phosphatidyl-choline synthesis, particularly by the lysolecithin acyltransferase pathway, may be related to a drug's effectiveness in decreasing serum lipids. We found that the transferase was moderately inhibited by the perfluorinated compounds. We also observed that this enzyme was virtually unaffected by 0.5 mM (Table VII) and also by 1 mM of clofibric acid. This agrees with the data reported by Parthasarathy et al. [27]. Our data do not support the hypothesis that reduced activity of lysolecothine acyltransferase plays a central role in the hypolipemic effect of the compounds we have tested. Inhibition of other enzymes of phospholipid synthesis may play a role.

Reduced release of lipids from the liver

In earlier studies, it has been reported that clofibrate reduces the release of lipids from the liver [13,14]. A direct effect on the excretion process by clofibrate was not confirmed by accumulation of liver triacylglycerols in clofibrate-fed, nor in PFOA-fed animals. A reduced release of lipids from liver was probably an effect of PFOSA, however, since the compound increased liver triacylglycerols by about 200% in spite of reduced rate of fatty acid synthesis (Table IV).

Since interference with the synthesis of cholesteryl ester may cause reduced hepatic lipid output [42], it may be concluded that reduction in cholesterol synthesis and esterification due to downregulation of HMG-CoA reductase and ACAT together with enhanced fatty acid oxidation in the liver, are effects caused by clofibric acid as well as by the perfluorinated compounds. This may reduce VLDL production by liver which plays a central role after the postprandial chylomicronemic period during which the liver is the dominating organ for delivery of lipids to serum.

In addition, the different hypolipemic drug may act by inhibiting the synthesis of other lipoprotein components such as phosphatidylcholine [27].

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Developmental changes in the expression of genes involved in cholesterol biosynthesis and lipid transport in human and rat fetal and neonatal livers

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Cloned cDNAs encoding a number of enzymes involved in cholesterol biosynthesis as well as extracellular and intracellular lipid transport were used to compare the developmental maturation of these biologic functions in the fetal and neonatal rat and human liver. The results of RNA blot hybridization analyses indicate that steady-state levels of rat HMG-CoA synthase, HMG-CoA reductase and prenyl transferase mRNAs are highest in late fetal life and undergo precipitous (up to 80-fold) co-ordinate reductions immediately after parturition. These changes reflect the ability of the fetal rat liver to produce large quantities of cholesterol as well as the repression of this function during the suckling period in response to exogenous dietary cholesterol. Striking co-ordinate patterns of HMG-CoA synthase, reductase and prenyl-transferase mRNA accumulation were also observed in four extrahepatic rat tissues (brain, lung, intestine and kidney) during the perinatal period. The concentrations of all three mRNAs in the 8-week-old human fetal liver are similar to those observed throughout subsequent intrauterine development with less than 2-fold changes noted between the 8th through 25th weeks of gestation. Analysis of the levels of human apo AI, apo AII, apo B and liver fatty acid binding protein mRNAs during this period and in newborn liver specimens also indicated less than 2–3-fold changes. These observations suggest that the 8-week human liver has achieved a high degree of biochemical differentiation with respect to functions involved in lipid metabolism/transport which may be comparable to that present in 19–21 day fetal rat liver. Further analysis of human and rat fetal liver RNAs using cloned cDNAs should permit construction of a developmental time scale correlating hepatic biochemical differentiation to be constructed between these two mammalian species.

Introduction

Marked changes in the requirements for products derived from isoprene (e.g. cholesterol) occur during development. The enzyme which catalyzes the key, rate-limiting step in cholesterol biosynthesis–microsomal 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase)–undergoes large fluctuations during rat liver ontogeny [1,2]. Enzyme activity is high prior to birth, declines to low levels during the suckling period (postnatal days 1–13) and demonstrates a transient increase at weaning. Changes in its activity parallel developmental alterations in the rate of incorporation of radiolabeled acetate into sterols [2]. Bruenger and Rilling [3] documented changes in the activities of two other cholesterogenic enzymes in the developing rat liver: squalene synthetase and prenyl transferase (or farnesyl/pyrophosphate synthetase). Prenyl transferase is one of five enzymes that participate in the conversion of mevalonate to squalene, the precursor of sterols. Both squalene synthetase and prenyl transferase display similar developmental activity profiles in rat liver: they fall following birth, rise to a peak during the mid to late suckling period (postnatal days 10–12), fall once more during the suckling-weaning transition reaching a nadir by postnatal day 20, only to rise one final time during weaning. The mechanisms which result in these alterations in enzyme activity may be quite complex as illustrated by the fact that the mevalonate-mediated

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decrease in HMG-CoA reductase levels observed in adult animals reflects decreases in gene transcription as well as increased rates of protein degradation [4,5].

Little is known about the developmental history of the activities of these enzymes in human fetal and neonatal liver or about the ontogeny of expression of genes involved in the transport and metabolic processing of lipids in this tissue. The rat liver provides a convenient reference for a comparative study of such developmental changes. For example in addition to the information about expression of cholesterogenic enzyme activities, recent analyses of apolipoprotein gene expression in the developing rat liver indicate that a complex pattern of activation occurs during late fetal and early neonatal life. Apolipoprotein AI and E mRNAs begin to accumulate in this tissue plus its embryonic homologue (the fetal yolk sac endoderm) between days 15–21 of the 21-day gestation period [6–9]. Remarkable increases in the levels of these mRNAs occur during the suckling period as the animals adapt to the high fat (principally triacylglycerol) diet of mothers milk [10,11]. By contrast, rat liver apo B mRNA levels reach a peak by the 18th fetal day that is not exceeded at any time during subsequent development [12]. Following birth, hepatic apo B mRNA concentrations progressively fall during the suckling and weaning periods [12]. This fall appears to be mediated by thyroxine [13]. A third pattern of activation is exhibited by the apo AIV gene which remains dormant until the suckling weaning transition (days 13–14) when the rat liver begins to export large amounts of triacylglycerol-rich lipoproteins [6].

We have begun a comparative analysis of the accumulation of mRNAs encoding proteins involved in lipid metabolism in the fetal and neonatal human and rat liver. A panel of cloned cDNAs encoding apolipoproteins AI, AII, and B, an intracellular fatty acid binding protein as well as HMG-CoA reductase, HMG-CoA synthase and prenyl transferase were used to characterize the state of enzymatic differentiation of the human fetal liver from weeks 8 to 25 of development and in the newborn. The results indicate that these mRNAs appear at an early phase of human fetal life (by week 8) and undergo only minimal (less than 3-fold changes) in their concentration in total liver RNA during the rest of intrauterine as well as early postnatal life. This early expression of lipid metabolic capability contrasts with the more marked changes in mRNA levels observed in the perinatal rat liver.

Materials and Methods

Preparation of RNA from rat and human liver

Timed-pregnant, neonatal and young adult Sprague-Dawley rats were obtained from Sasco (St. Louis, MO). Weaned animals were maintained on a standard chow diet ad libitum and a fixed 12 h (6:00 a.m. to 6:00 p.m.) light cycle. Animals (n = 10–40 for each time point) were killed between 1200 and 1400 h and their livers, lungs, brains, kidneys and small intestines immediately frozen in liquid nitrogen. Human fetal liver samples from first and second trimester aborted fetuses were obtained by Schwartz et al. [14] and maintained at −90°C for 15–20 years. Fetal age was estimated from crown-rump lengths using nomograms developed by Tanimura et al. [15]. Breitfeld and Schwartz [16] and Michaelson and Orkin [17] have used these human fetal liver samples previously to successfully prepare RNA for in vitro translation. Additional human liver samples were obtained from a preterm newborn and a full term newborn, both of whom died of acute respiratory failure. These were stored at −90°C for about 15 years [14]. A single adult liver specimen was procured from a male organ donor who died of trauma and had no history or clinical evidence of hepatic dysfunction [18].

Total cellular RNA was extracted from frozen pulverized tissues using the guanidine thiocyanate/cesium chloride procedure [19]. RNA integrity was assessed by denaturing, methylmercury agarose gel electrophoresis [20].

RNA blot hybridization studies

Dot blots were prepared by applying four amounts of each tissue RNA sample (0.5, 1, 2, and 3 μg) to nitrocellulose filters as described in a previous publication [6]. Yeast tRNA was added to each tissue RNA sample prior to denaturation so that the total RNA input per dot was always 3 μg. Blots containing samples of rat liver, intestine, kidney, brain and lung RNA were probed with ^{32}P-labeled, double-stranded cDNAs encoding hamster HMG-CoA reductase [22], rat HMG-CoA synthase [23], and rat prenyl transferase [24]. Dot blots of human liver RNA samples were probed with ^{32}P-labeled cDNAs specifying human preproapo AI [25], human preproapo AII [26], human preapo B [27], human liver fatty acid binding protein [28], human α-fetoprotein [29], human HMG-CoA reductase [30], rat prenyl transferase and rat HMG-CoA synthase. Conditions selected for filter hybridization and washing are listed in Ref. 12. These stringencies were equivalent to those used by others to produce specific interactions between these cDNAs and their respective mRNAs. The relative abundance of each mRNA in the tissue RNA preparations was determined by quantitative scanning laser densitometry of filter autoradiographs using an LKB XL Ultroscan densitometer. Only signals in the linear range of film sensitivity were utilized for calculations of relative mRNA concentration.

Northern blots of total cellular RNA were produced following electrophoretic fractionation through agarose gels containing formaldehyde [31].

Results and Discussion

Accumulation of HMG-CoA synthase, HMG-CoA reductase and prenyl transferase mRNAs in the developing rat liver

Cloned cDNAs encoding HMG-CoA synthase, HMG-CoA reductase, and prenyl transferase were used to probe dot blots of total cellular RNA prepared from rat livers harvested during fetal days 16–21, the suckling period (defined as the first 13 postnatal days, Ref. 32), the weaning phase (days 14–28) and from animals proceeding through sexual maturation (postnatal days 35–70). Material was collected from 10 to 40 male and female animals representing 1–4 litters at each developmental stage studied. The results of our dot blot hybridization analyses are presented in Fig. 1 and show

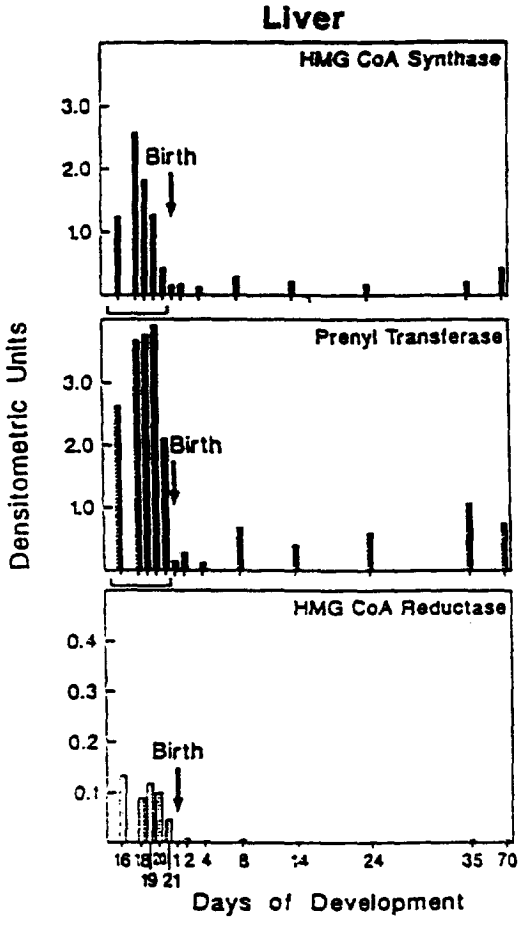

Fig. 1. Developmental changes in rat liver HMG-CoA Synthase, HMG-CoA Reductase and Prenyl Transferase mRNA levels. Total cellular RNA was prepared from pooled rat livers (n = 10–40 animals per time point). Cloned rat cDNAs encoding the three enzymes were used to probe dot blots containing four concentrations of each RNA. The relative concentration of each mRNA was calculated based on scanning laser densitometry of filter autoradiographs and expressed in arbitrary densitometric units. Note that the specific activities of the probes were not identical and therefore no comparisons can be made about the relative levels of each mRNA at a particular stage of liver development.

a remarkable similarity in the patterns of change of each mRNA during rat fetal and neonatal development. High levels are noted during late gestation. These fall abruptly within 24 h after parturition reaching concentrations that are as much as 80-fold lower than those peak levels encountered prior to birth (e.g., see the middle panel of Fig. 1 which shows changes in prenyl transferase mRNA concentration). A transient 2–4-fold rise in HMG-CoA synthase and prenyl transferase but not HMG-CoA reductase mRNA accumulation occurs during the mid-suckling period (day 8) followed by yet another rise during early adulthood. It is important to note that this pattern of change is not a general phenomenon in the developing rat liver. For example, when the developmental profiles of apo AI, apo AIV and L-FABP mRNAs were studied using these RNAs, quite different ontologic changes were noted (see, for example, Ref. 6).

The developmental profile of rat liver HMG-CoA reductase and prenyl transferase mRNAs parallel the changes in the activities of these enzymes which have been previously documented by several groups [3,33–35]. The corresponding activities of HMG-CoA synthase have not been reported. The rat fetus obtains only about 10% of the sterol required for growth and development from its mother [36]. Therefore, it is not surprising that very high levels of these mRNAs are observed in rat fetal liver during the period surveyed. The highest levels of apo B mRNA occur during the same phase of rat liver development (i.e., in late gestation) only to be followed by an abrupt, early postnatal drop [12]. This provision for expressing the principal apolipoprotein involved in cholesterol transport at a time of maximal endogenous production emphasizes the co-ordinate nature of these developmental changes in the fetal rat liver.

The precise signals for the rapid postnatal fall in the capacity of the livers of suckling rats to synthesize cholesterol are not known. It may represent an adaptive response to adequate supplies of exogenous cholesterol delivered via mother's milk [3,33,37]. Based on the RNA blot hybridization data, it appears that the mechanism involves at least in part a reduction in the steady-state levels of mRNAs encoding these key enzymes in cholesterol biosynthesis. The data do not allow us to say to what extent such alterations reflect a change in gene transcription or mRNA stability.

The changes observed in the levels of prenyl transferase mRNA during the late suckling through weaning phases can be directly correlated with changes in the levels of this enzyme activity [3]. By the 14th postnatal day, HMG-CoA reductase activity in rat liver has fallen to nearly undetectable levels [36]. However, during this second postnatal week, rises in prenyl transferase occur. This rise coincides with that exhibited by another enzyme involved in the mevalonate to squalene pathway.

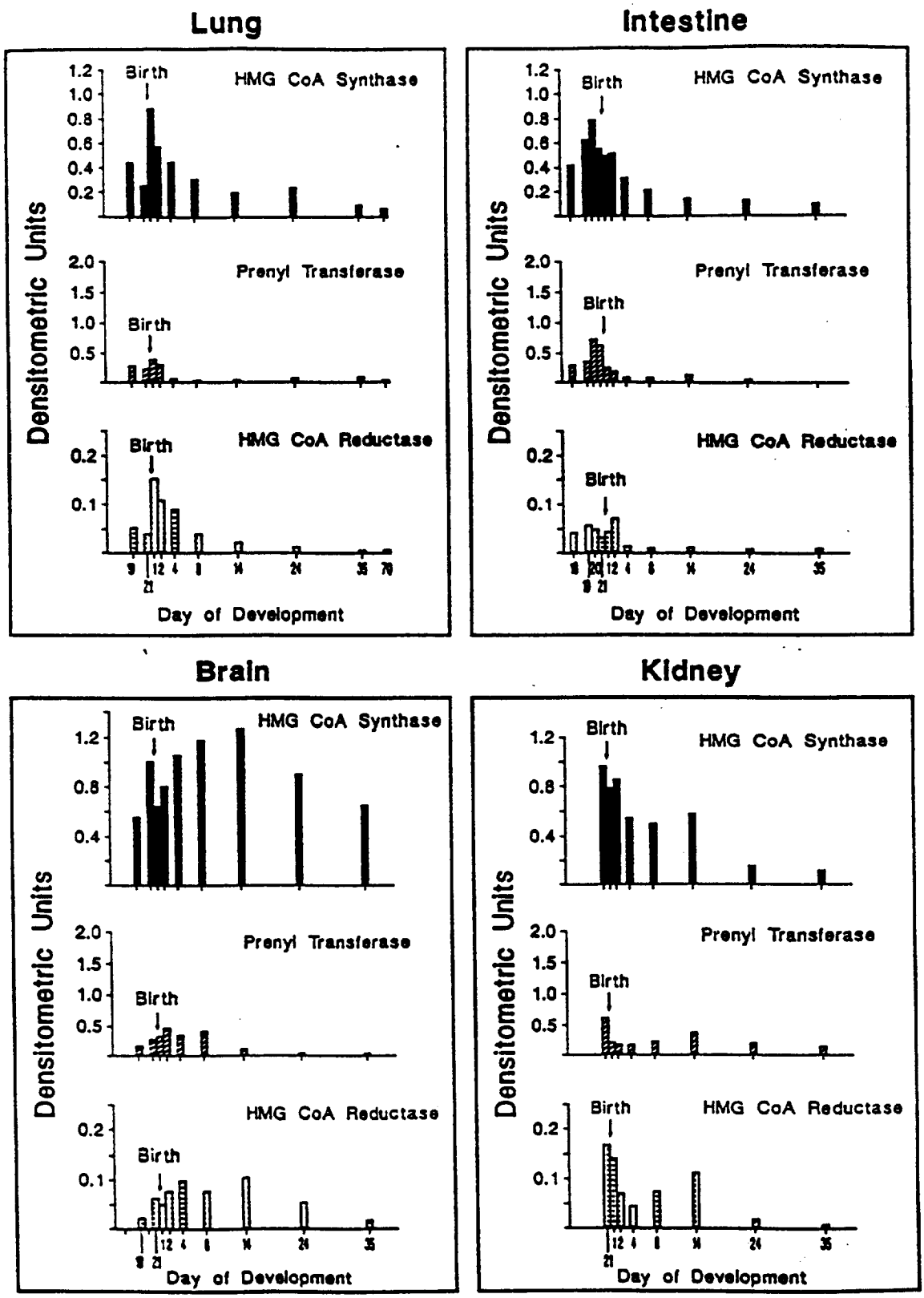

Fig. 2. Coordinate patterns of accumulation of HMG-CoA synthase, prenyl transferase, and HMG-CoA reductase mRNAs during lung, intestinal, brain and kidney development. Total cellular RNA was isolated from pooled tissues harvested from 10–40 rats at each day of fetal and postnatal life noted on the *x*-axis. Relative mRNA concentrations were determined by scanning dot blot autoradiographs and expressed in arbitrary densitometric units. The only comparison which is permitted by this form of data expression is that which involves the same mRNA within a given tissue.

squalene synthetase. These changes in prenyl transferase and squalene synthetase activity do not correspond to any known change in cholesterol synthesis. Bruenger and Rilling [3] noted that since the primary metabolic destination of isoprenoid precursors is cholesterol, changes in the activities of these enzymes may reflect as yet unknown developmental alterations in the metabolic targeting of mevalonate to other compounds (e.g., dolichol and its derivatives, ubiquinones, or isopentenyl tRNAs [38] and prenylated proteins [39]). Although information about the activity profile of HMG-CoA synthase during rat liver development has not been documented in the literature, based on the mRNA data presented in Fig. 1, its developmental profile would be predicted to more closely resemble that of prenyl transferase than HMG-CoA reductase. Moreover, the blot hybridization studies demonstrate for the first time that there are co-ordinate developmental changes in the levels of these three mRNAs encoding enzymes involved in cholesterol biosynthesis.

Expression of the HMG-CoA synthase, HMG-CoA reductase and prenyl transferase genes in extrahepatic tissues during rat development

The remarkable similarity in the accumulation 'profiles' of these three mRNAs observed during the perinatal period of rat liver differentiation was not unique to this organ. This is illustrated in Fig. 2 which summarizes results obtained from probing dot blots of lung, small intestinal, brain and kidney RNAs prepared from 10–40 animals at each of as many as ten different stages of development. Two obvious conclusions can be made after inspection of the data. First, the timing and direction of developmental variation in relative mRNA concentration was virtually identical for each species within each tissue. Second, as in the liver, the highest concentration of each mRNA during lung, intestinal and kidney development was encountered in the late and early postpartum period with subsequent declines occurring in the suckling and/or weaning phases. The notable exception was brain where a progressive postnatal rise in the concentration of each mRNA occurred that generally reached a peak in the mid to late suckling period. Thus expression of the rat HMG-CoA synthase, HMG-CoA reductase and prenyl transferase genes appears to be elaborately programmed to respond in a similar temporal fashion both within and between different tissues from late fetal life through the end of weaning.

Accumulation of mRNAs encoding cholesterol biosynthetic enzymes and lipid transport proteins in fetal, neonatal and adult human liver

Fig. 3 provides the results of our analysis of developmental changes in the concentration of the three cholesterol biosynthetic enzyme mRNAs in human liver. A cloned human HMG-CoA reductase cDNA plus cDNAs encoding rat HMG-CoA synthase and rat prenyl transferase were used for these studies. Since the last two represent heterologous cDNAs, a preliminary experiment was performed. Northern blots of RNA prepared from a human hepatocellular carcinoma cell line (Hep G2) were probed with the rat cDNAs employing the same hybridization stringencies listed in Ref. 12 but the final wash temperature was reduced from 55°C to 45°C. The results indicated that each rat probe reacted with a unique human mRNA species–3.1 and 2.2 kb HMG-CoA synthase mRNA and a 1.2 kb prenyl transferase mRNA (data not shown). These sizes are comparable to those previously reported for the corresponding rat mRNAs [23,40]. Dot blots of total cellular RNA isolated from 8.5–25-week fetal livers ($n = 1$–3 individuals per time point) plus two newborns and one adult without evidence of liver dysfunction were then prepared and probed with the three cDNAs using hybridization and washing conditions established above. As in the case of the rat liver RNA dot blots, multiple concentrations (0.5–2 μg) of each human liver RNA sample were included in the dot blots (see panel A of Fig. 3).

Inspection of Fig. 3B reveals a relatively monotonous developmental profile for all three mRNAs. By 8 weeks of gestation these mRNAs have achieved steady-state levels which do not change more than 2-fold during the remaining 17 weeks of fetal life that were surveyed and less than 3–5-fold when compared to the two newborn and single adult RNA preparations. When there was an opportunity to compare more than one sample at a given stage of human fetal development, remarkably little individual variation was noted in the relative level of a given mRNA.

This less than 2-fold change in cholesterogenic mRNA levels during fetal life is not a general phenomena. It contrasts with results obtained when these same RNA preparations were probed with other cDNAs encoding proteins not involved in lipid metabolism. For example, a control experiment which examined the levels of α-fetoprotein mRNA disclosed the approx. 10-fold reduction expected between first trimester liver and liver harvested at the beginning of the last trimester (see panels A and B of Fig. 3) [41]. In addition, a recent study revealed marked changes in the levels of epsilon, gamma and theta globin mRNAs as well as ceruloplasmin mRNA in the 10–25-week human fetal liver RNA samples [42]. Moreover, the patterns of change of these five different mRNAs were quite distinct from one another.

There is little information about the activities of these cholesterol biosynthetic enzymes during human fetal development. However, the relatively monotonous HMG-CoA reductase, HMG-CoA synthase and prenyl transferase mRNA levels are compatible with observa-

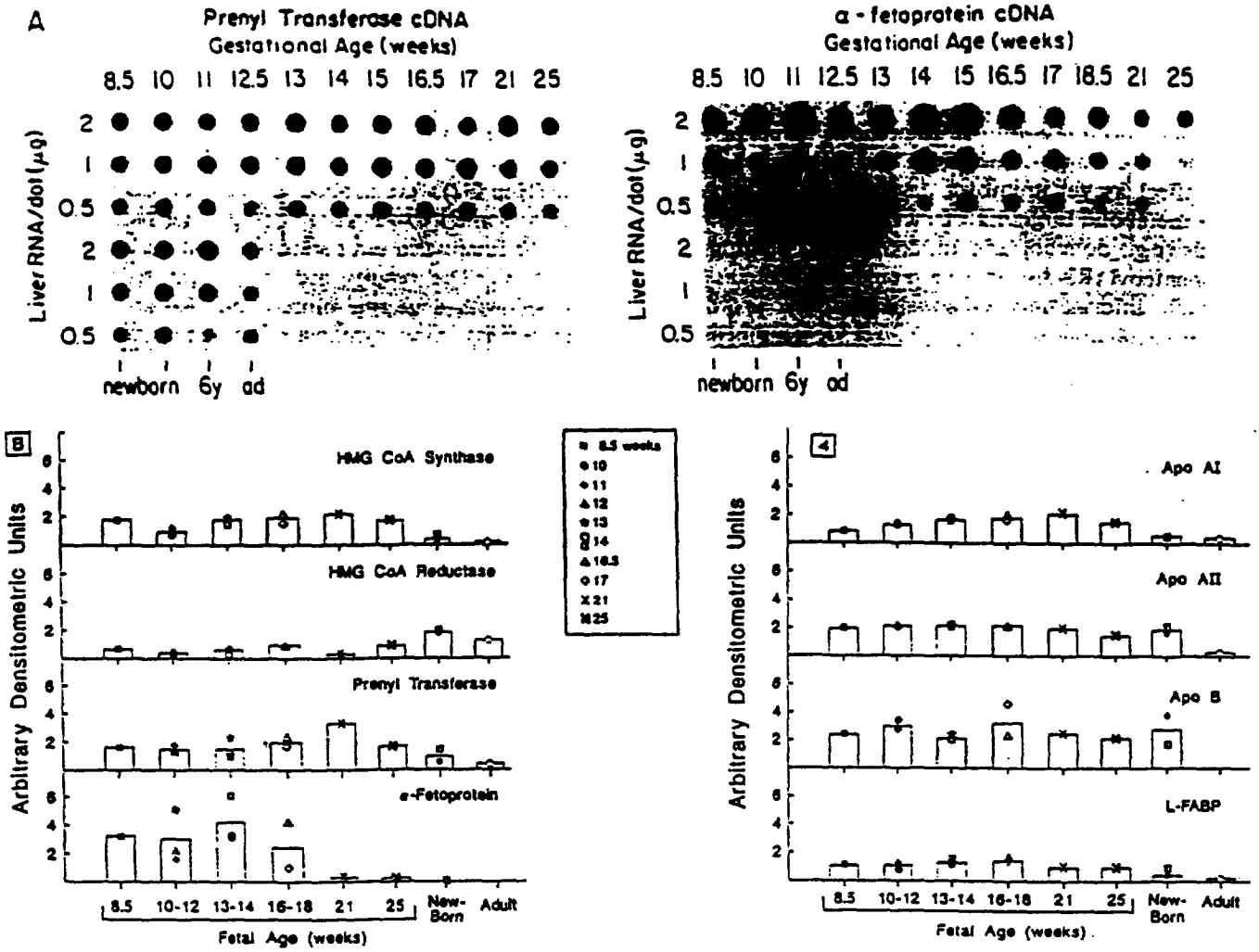

Fig. 3. Developmental changes in the concentration of mRNAs encoding cholesterogenic enzymes in samples of human fetal liver. cDNAs encoding human HMG-CoA reductase plus rat HMG-CoA synthase, rat prenyl transferase (plus human α-fetoprotein) were used to probe dot blots containing several concentrations (0.5–2 μg) of each human liver total cellular RNA sample. Fetal age is based on crown-rump lengths [15]. Fetal age is based on crown-rump lengths [15]. Note that the two 14-week samples came from separate fetuses (see the key defining the symbol ascribed to each RNA preparation). Panel A displays representative dot blots probed with ^{32}P-labeled prenyl transferase and α-fetoprotein cDNAs. Panel B presents developmental profiles of mRNAs encoding the three cholesterol biosynthetic enzymes and α-fetoprotein. As in Fig. 1 arbitrary densitometric units were used to express mRNA levels. No conclusions can be made about the relative concentrations of each of the four mRNAs in a liver RNA sample prepared at a particular developmental stage.

Fig. 4. Developmental profile of human liver mRNAs specifying proteins involved in lipid transport. Blots prepared with the same fetal, neonatal and adult human liver RNA samples as those used to generate the data shown in Fig. 3, were probed with ^{32}P-labeled cloned cDNA specifying human apolipoproteins apo AI, apo AII, apo B, and liver fatty acid binding protein (L-FABP).

tions made by Carr and Simpson [43] that only slight alterations occur in the rate of cholesterol synthesis in the developing human fetal liver. (This rate was estimated to be approx. 9 mg/day during the 18th week of gestation.)

When the levels of other mRNAs encoding proteins involved in lipid transport were examined in human fetal liver, similar 'flat' developmental profiles were observed (Fig. 4). mRNAs specifying the two principal human HDL apolipoproteins, AI and AII, plus the principal protein component of LDL, apo B, achieve steady-state concentrations by 8 weeks of gestation that are similar to those documented during weeks 10–25 and in term infants. Variations were less than 2-fold in all cases. Moreover, the mRNA encoding liver fatty acid binding protein (L-FABP), a small cytoplasmic protein believed to participate in the uptake and/or metabolic processing of exogenous fatty acids (reviewed in Ref. 44), displays a similar lack of change in its concentration during this period (Fig. 4). Together these data

suggest an early biochemical maturation of the human fetal liver with respect to its capacity for cholesterol synthesis and lipid transport.

Differentiation of the human and rat fetal liver

There are very few studies which have provided information about the biochemical maturity of human liver from midgestation to term, so it is very difficult to put our data concerning expression of genes involved in lipid metabolism in any sort of comparative metabolic context at the present time. Greengard has conducted an extensive analysis of the literature dealing with enzymatic differentiation of human and rat liver [45]. Although the data for human are both limited in the number of enzymes examined and the scope of the analyses through fetal life, based on published reports concerning approx. 30 enzymes it appears that most liver enzymes exhibit significant quantitative differences in their activities between the second trimester and adulthood. For example, enzymes involved in synthesis of DNA, pentoses, nonessential amino acids, as well as glycolysis have activities in the actively growing fetal liver which are different from those in the adult tissue. In the few examples where developmental profiles are available in fetal and newborn human and rat liver, it appears that enzyme activities change in the same direction in both mammalian species [45] although the timing of such changes has not been well enough characterized to provide a comparative time scale for human and rat liver differentiation. Comparison of our observations concerning human apo AI, apo B and L-FABP mRNA levels in total human fetal liver RNA with previously published studies in rat liver [6,12,46] suggests that the 8–12-week human fetal liver is at least as well 'differentiated' as the 19–21-day fetal rat liver with respect to expression of these mRNAs. Further studies, based on RNA hybridization techniques, should prove valuable in establishing this comparative developmental time scale for a variety of differentiated functions.

One important caveat needs to be mentioned concerning interpretation of these data. Total cellular RNA prepared from fetal rat and human liver by definition includes mRNAs from all cellular constituents in the liver. These cellular populations undergo remarkable changes during development. A limited number of quantitative morphometric studies of human fetal liver indicate that hepatic parenchymal cells account for approx. 50–70% of its cellular population between the 8th to 28th weeks of gestation with erythroid cells representing 40–50% of fetal liver nuclei and granulocytic precursors less than 10% [47]. In the fetal rat liver, approx. 50% of liver cells are involved in hematopoiesis just prior to parturition with this number rapidly decreasing to less than 5% by the end of the suckling period [48]. The consequences of these shifts in cell populations are obviously important when considering regulation of gene expression in this tissue. For example, the relatively constant levels of mRNAs encoding proteins involved in lipid metabolism that were documented in total human fetal liver RNA during gestation may 'mask' relatively dramatic changes in their concentrations within a given cell type and/or changes in their expression in different cell types. Therefore, it will be very important to extend these analyses using in situ hybridization and immunocytochemical techniques to examine changes in the cell specific expression of these genes in the fetal human and rat liver.

Acknowledgements

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3M SPECIALTY ADHESIVES & CHEMICALS ANALYTICAL LABORATORY

Request #'s **53030**

To: Leo Gehlhoff - (309727) -3M Chemicals - 236-2A-01

From: Tom Kestner - (3-5633) SA&C Analytical Lab - 236-2B-11

Subject: ***Fluorochemical Isomer Distribution by ^{19}F-NMR Spectroscopy***

Date: December 1, 1997

SAMPLE DESCRIPTIONS:

- FC-95, lot # 217 (T-6295); Nominal product = C_8F_{17}-SO_3(-) K(+)

INTRODUCTION:

This sample was subjected to a ^{19}F-NMR spectral analysis method to determine the identities and relative concentrations of the fluorochemical isomers and as many other identifiable impurity components as possible.

EXPERIMENTAL:

A portion of the sample solid was totally dissolved in DMSO-d_6 and then a 376 MHz ^{19}F-NMR spectrum (F53030.401) was acquired using a Varian UNITYplus 400 FT-NMR spectrometer. Aida Robbins prepared the sample for analysis and she also acquired and plotted the NMR spectrum.

RESULTS:

The ^{19}F-NMR spectrum was used to determine the identities and relative concentrations of the nominal fluorochemical isomers and three other impurity components in this sample. The qualitative and quantitative compositional results which were derived from the single trial ^{19}F-NMR spectral analysis are summarized in **TABLE-1** on the following page.

A copy of the NMR spectrum and the spectral assignments data page are attached for your reference. If you have any questions about these NMR results, please let me know. I apologize for the delay in completing this work.

Tom Kestner

c: Jim Johnson - EE&PC - 2-3E-09

File Reference: LG53030.DOC/43

TABLE-1
Sample: T-6295 (FC-95, Lot 217)
^{19}F-NMR Compositional Results

Structural Assignments	^{19}F-NMR Relative Mole% Concentrations
$CF_3(CF_2)_x$-SO_3(-) K(+) (Normal chain, where x is mainly 7)	70.0%
$CF_3(CF_2)_x$-$CF(CF_3)$-$(CF_2)_y$- SO_3(-) K(+) (Internal monomethyl branch, where x+y is mainly 5, and $x \neq 0$, $y \neq 0$)	17.0%
$(CF_3)_2CF$-$(CF_2)_x$- SO_3(-) K(+) (Isopropyl branch, where x is mainly 5)	10.3%
C_xF_{2x+1}-$CF(CF_3)$- SO_3(-) K(+) (Alpha branch, where x is mainly 6)	1.6%
R_f-CF_2-SF_4-F	0.35%
$(CF_3)_3C$-$(CF_2)_x$- SO_3(-) K(+) (t-butyl branch, where x is mainly 4)	0.23%
CF_3-$(CF_2)_x$-$C(CF_3)_2$-$(CF_2)_y$- SO_3(-) K(+) (Internal gem-dimethyl branch, where x+y is mainly 4, and $x \neq 0$)	0.15%
Possible CF_3-SO_3(-) K(+)	0.25%
Possible CF_3-CO_2(-) K(+)	0.05%