Induction of peroxisomal enzymes in livers of neonatal rats exposed to lactating mothers treated with hypolipidaemic drugs

Role of drug metabolite transfer in milk

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Lactating rats were administered by gavage 100 mg/kg body wt. twice a day of either nafenopin or Wy-14,643, two hypolipidaemic drugs with hepatic peroxisome proliferative property. Neonatal rats, after feeding from the drug-treated mothers for 8–14 days, showed sustained increases in both the proliferation of hepatic peroxisomes, as well as in levels of the peroxisome-associated enzymes catalase (3-fold), carnitine acetyltransferase (15–35-fold), peroxisomal enoyl-CoA hydratase (29–46-fold), and palmitoyl-CoA oxidation (12–14-fold). These increases in enzyme activities in suckling rats were similar to those seen in the livers of the drug-treated, lactating mothers after 14 days of treatment. After administering [^3H]nafenopin or [^3H]Wy-14,643 to lactating rats, significant levels of drug-derived radioactivity were observed in suckling rat gastric milk curds by 2–4 h with significant radioactivity seen in suckling rat livers by 4–6 h. T.l.c. analysis of organic extracts of milk samples from [^3H]Wy-14,643 treated animals indicated no detectable levels of the parent drug, only more-polar metabolites. Wy-14,643 metabolites preparatively purified from a rat liver microsomal fraction incubation induced peroxisome proliferation when injected into a neonatal rat. Preparative high pressure liquid chromatography purification and mass spectral analysis has allowed preliminary assessment of the structures of the Wy-14,643 microsomal metabolites. It is concluded that one or more of the metabolite fractions of Wy-14,643 transferred in milk exert the biological ability to induce peroxisome proliferation and peroxisomal enzymes in neonatal livers.

Exposure of rodents to any of a group of structurally-dissimilar drugs which are capable of lowering plasma triglyceride levels is uniformly associated with a marked proliferation of peroxisomes in hepatic parenchymal cells (Hess *et al.*, 1965; Svoboda *et al.*, 1967; Reddy & Krishnanantha, 1975). This increase in liver peroxisome content is also seen after exposure of animals to commonly used industrial plasticizers such as di-(2-ethylhexyl)phthalate (Reddy *et al.*, 1976a; Moody & Reddy, 1978; Osumi & Hashimoto, 1978). These cytoplasmic organelles contain catalase, several hydrogen peroxide-generating oxi-

dases, carnitine acetyltransferase (Markwell *et al.*, 1973) and several enzymes involved in the β -oxidation of long-chain fatty acids (Lazarow & de Duve, 1976). The activities of catalase, carnitine acetyl transferase and peroxisomal fatty acid β -oxidation system in liver are elevated in association with peroxisome proliferation (Reddy *et al.*, 1982; Lazarow, 1977; Leighton *et al.*, 1975; Moody & Reddy, 1978), and these increases are associated grossly with hepatomegaly in the hypolipidaemic drug-treated rodents. Clofibrate, and other hypolipidaemic aryloxyalkanoic acids such as gemfibrozil, fenofibrate, nafenopin and methyl clofenapate, as well as structurally unrelated hypolipidaemic drugs, such as Wy-14,643, BR-931 and tibrice acid, have been shown to produce hepatocellular tumours in both mice and rats when chronically included in the diet (J. K. Reddy *et al.*, 1976b, 1979, 1980; Fitzgerald *et al.*, 1981). Because

Abbreviations used: SDS, sodium dodecyl sulphate; Wy-14,643, [4-chloro-6-(2,3-xylidino)-2-pyrimidinyl-thio]acetic acid; nafenopin, 2-methyl-2-[*p*-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]propionic acid; PPA-80, peroxisome-proliferation-associated polypeptide of M_r 80 000; h.p.l.c., high pressure liquid chromatography.

of the long-term administration of drugs such as clofibrate for the control of hyperlipidaemic states in humans, it is essential to examine the biochemical results of persistent peroxisome proliferation and the observed coupling of this chronic phenomenon to an eventual sequel, the induction of hepatic tumours. In view of the widespread use of hypolipidaemic drugs and industrial plasticizers, which increase peroxisomal enzymes, a careful examination of the biochemical effects of these compounds in foetal and neonatal systems appears relevant.

The objectives of the work described here were to: (a) determine whether short-term peroxisome-associated effects occurred in neonatal rats which were nursing from mothers consuming the hypolipidaemic drugs nafenopin and Wy-14,643, and (b) determine the pharmacological disposition of these model compounds in this animal system, measuring the degree of transfer of these drugs or their biologically active metabolites via milk to the neonatal animals.

Experimental

Chemicals

The hypolipidaemic compound Wy-14,643 was obtained from Wyeth Laboratories, Radnor, PA, U.S.A. and nafenopin was supplied by Ciba-Geigy Corporation, Summit, NJ, U.S.A.

[³H]Nafenopin (9.9 mCi/mmol) was obtained from Dr. Neal Handly, California Institute of Technology, Pasadena, CA, U.S.A. [³H]Wy-14,643 (109.6 mCi/mmol) was custom synthesized by New England Nuclear Corporation, Boston, MA, U.S.A. [1-¹⁴C]Palmitoyl-CoA (59 mCi/mmol) was obtained from Amersham, Arlington Heights, IL, U.S.A.

Crotonyl-CoA, NAD, CoA, palmitoyl-CoA, glucose 6-phosphate, glucose-6-phosphate dehydrogenase and bovine serum albumin were obtained from Sigma.

Purified acrylamide, *NN'*-methylenebisacrylamide, dithiothreitol and t.l.c. plates (100 μ m silica gel) were purchased from Eastman Kodak. The solvents for chromatography were either spectrograde or h.p.l.c. grade.

Animals

F344 pregnant rats weighing about 180 g were purchased from A. R. Schmidt/Sprague-Dawley, Madison, WI, U.S.A. After parturition the litters were decreased to four or six in number (equal numbers of each sex). The mothers were fed by gavage with the hypolipidaemic compounds nafenopin or Wy-14,643 (100 mg/kg body wt. twice a day) after delivery. Dimethyl sulphoxide was used to dissolve the chemicals (10%, w/v). For radiotracer experiments, mothers were given either 100 μ Ci of [³H]Wy-14,643 or [³H]nafenopin by gavage and

neonates consumed milk for the specified periods. Neonates in the control group consumed milk from mothers given dimethyl sulphoxide only.

Enzyme assays

The enzymes catalase (EC 1.11.1.6) and carnitine acetyltransferase (EC 2.3.1.7) were assayed in liver homogenates as described previously (Reddy *et al.*, 1981). The palmitoyl-CoA oxidizing activity was measured as described by Lazarow (1981) using [1-¹⁴C]palmitoyl-CoA as substrate. Enoyl-CoA hydratase (EC 4.2.1.17) activity was assayed using 200 μ M-crotonyl-CoA as substrate in 0.3 M-Tris/HCl, pH 7.4, containing 5 mM-EDTA and 0.05 mg of bovine serum albumin/ml in a 1 ml mixture. The heat-labile enoyl-CoA hydratase activity was inactivated when the homogenates, diluted with 50 mM-potassium phosphate, pH 7.0, were heated at 57°C for 5 min (Osumi & Hashimoto, 1979).

Subcellular fractionation

The livers from male and female suckling rats of each litter were pooled and homogenized (10%, w/v) in 0.25 M-sucrose containing 0.1% ethanol, using a Potter-Elvehjem homogenizer. The large-particle and microsomal fractions were prepared as described previously (M. K. Reddy *et al.*, 1980). Protein concentrations were determined by the method of Lowry *et al.* (1951).

Drug metabolism studies

For studies of [³H]Wy-14,643 metabolism, 2 ml incubation mixtures contained 100 nmol of [³H]-Wy-14,643 (20 mCi/mmol in 50 μ l of acetone), 14.0 μ mol of glucose 6-phosphate, 0.2 unit of glucose-6-phosphate dehydrogenase, 1.0 μ mol of NADP⁺, 6.0 μ mol of MgCl₂ and 100 μ mol of potassium phosphate (pH 7.4). The mixtures were incubated at 37°C for 30 min with 1.0 mg of liver microsomes or large-particle fraction obtained from normal or Wy-14,643-treated rats. The blank tube contained boiled enzyme. The reaction mixture was extracted once with 5 vol. of ethyl acetate. The organic phase was evaporated and the residue was resuspended in methanol for spotting on the t.l.c. plates. Ethyl acetate extracted >99% (in boiled enzyme blanks) or 72% (non-boiled enzyme incubations) of the radioactivity from reaction mixtures after a 30 min incubation.

Wy-14,643 metabolites, in amounts adequate for studies of their peroxisome-inducing activity, were prepared by using a large-scale microsome incubation. The 40 ml incubation contained 3200 nmol of Wy-14,643; a trace amount (8 μ Ci) of [³H]-Wy-14,643 was also added to aid in locating the metabolites on t.l.c. plates after chromatography. The microsomes and cofactor components were proportionately increased from the above 2 ml

incubation. Some 2300nmol of Wy-14,643 metabolites (assuming the starting specific activity) were eluted and recovered from silica which had been scraped from t.l.c. plates (between 1.5 and 4.0cm from bottom; see Fig. 4c). This metabolite fraction was dissolved in methanol/0.9% saline (1:4, v/v) and used for direct injection into a neonatal rat.

Analysis of milk samples

Neonatal rats were killed after specified times of consuming milk from mothers previously dosed with [^3H]Wy-14,643 or [^3H]nafenopin. Solid milk curds were removed from neonatal rat stomachs, weighed and homogenized in 4 vol. of 0.1M-potassium phosphate buffer, pH 7.4. The ^3H content of the samples was determined by liquid-scintillation spectrometry. For [^3H]Wy-14,643-treated animals, the milk samples from all of the time points were pooled and extracted with ethyl acetate. The organic phase was transferred and evaporated, leaving an oily residue. Aliquots of this oil were spotted and analysed by t.l.c.

Chromatography

Silica gel plates (Eastman Chromatogram) were prechromatographed in chloroform/ acetic acid (99.9:0.1) and dried. Milk samples (5 μl of oil) or liver drug metabolism reaction products (in 25 μl of methanol) were spotted on to t.l.c. strips (20cm $\times$ 4cm) and developed in the above solvent system. Dried strips were cut into 0.5cm $\times$ 4cm strips, each of which was placed into a 15mm $\times$ 45mm mini-scintillation vial with 4.0ml of 'cocktail' and counted.

For mass spectral analysis, two Wy-14,643 metabolite fractions were purified and collected by h.p.l.c. Extracts of Wy-14,643 microsomal incubations (see above) were applied to a reverse phase column (4.6mm $\times$ 25cm; Altech) and eluted with a linear, 100min gradient of methanol/water/ acetic acid (500:499: 1, by vol.) to methanol. The initial flow rate was 1ml/min, and fractions were collected at 30s intervals. Approx. 500nmol of each of the two Wy-14,643 metabolite fractions were separated by h.p.l.c. and then collected and concentrated in acid-cleaned glassware prior to mass spectral analysis. The 50ml microsomal incubation from which the metabolites were isolated received only unlabelled Wy-14,643 as substrate. Mass spectral fragmentation analysis was done on a Hewlett-Packard 5985 mass spectrometer.

SDS/polyacrylamide-gel electrophoresis

Electrophoresis was performed on polyacrylamide slab gels by the method of Laemmli (1970). Polyacrylamide gels (7.5%) were prepared from a stock of 30% acrylamide and 0.8% *NN'*-methylenebisacrylamide as described previously (Reddy & Kumar, 1977). The stacking gel was 4.5% acryl-

amide with 125mM-Tris/HCl (pH 6.8)/0.1% SDS/0.024% tetramethylethylenediamine/0.1% ammonium persulphate. The electrode buffer used was 25mM-Tris/glycine, pH 8.3, which contained 0.1% SDS. The samples of subcellular fractions were solubilized in a solution containing 20% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) mercaptoethanol and 0.001% Bromophenol Blue in 65mM-Tris/HCl, pH 6.8, by heating for 3min in boiling water (Laemmli, 1970). Aliquots of dissolved protein (20 μg) were loaded onto a stacking gel and electrophoresed at 30mA/slab until the marker dye had reached the bottom of the gel. The gels were stained with 0.1% Brilliant Blue R and destained as described previously (Lalwani *et al.*, 1981).

Electron microscopy

The neonatal rat livers were minced into blocks of 1mm³ in 2.5% glutaraldehyde in 0.1M-sodium cacodylate buffer, pH 7.4, and fixed for 30min, washed and post-fixed in 1% OsO₄ in 0.1M-scollidine buffer, pH 7.4, for 1h at 4°C, dehydrated and embedded in Epon. Thin sections were cut and examined in a Hitachi-HU12 electron microscope. Selected samples of liver were processed for the cytochemical localization of peroxisomal catalase by the alkaline 3,3'-diaminobenzidine method of Novikoff & Goldfischer (1969).

Results and discussion

Induction of neonatal liver peroxisomal enzymes

The time course for induction of peroxisomal catalase and carnitine acetyltransferase in the livers of neonatal rats feeding from nafenopin-treated, lactating mothers is illustrated in Figs. 1(a) and 1(b). Since there was no discernible sex difference in the activities of these enzymes the data are presented as the mean $\pm$ s.d. for both sexes. For both enzymes, activities were markedly elevated (4- and 20-fold) above controls by 7 days, which is similar to the time course of induction for catalase and carnitine acetyltransferase seen in adult rodents fed either nafenopin or other hypolipidaemic drugs (Reddy *et al.*, 1982). Stäubli *et al.* (1977) reported a similar increase in hepatic catalase activity when nafenopin was administered by intubation, daily for five consecutive days, to newborn rats from the 5th day post partum. The magnitude of catalase induction in the neonate is either the same or greater than that seen in the livers of the lactating mothers from which the neonates fed (Table 1). The results presented in Table 1 also clearly indicate that two peroxisomal enzyme activities indicative of peroxisomal β -oxidation of fatty acids, enoyl-CoA hydratase and palmitoyl-CoA oxidation (Lazarow & de Duve, 1976), are dramatically increased in neonate livers after a 14 day exposure to lactating mothers to

Fig. 1. Change in peroxisomal enzyme levels in livers of neonatal rats nursing from mothers injected with the hypolipidaemic drug nafenopin

Lactating mother rats received nafenopin (100 mg/kg, twice daily) by gavage beginning on the day of birth. Neonatal rats were given free access to control (untreated) or nafenopin-treated lactating rats for the specified number of days. Neonatal animals were sacrificed and enzyme activities were determined in the liver homogenates (see the Experimental section). (a), Catalase activity; (b) carnitine acetyltransferase activity. (●), Control (livers from neonates exposed to untreated lactating mothers); (○), nafenopin-treated (livers from neonates exposed to nafenopin-treated lactating mothers).

Table 1. Change in peroxisomal enzyme levels in the livers of nursing neonatal rats or lactating mother rats which were exposed to either of the hypolipidaemic drugs nafenopin or Wy-14,643

Lactating rats received nafenopin or Wy-14,643 (100 mg/kg, twice daily) by gavage beginning on the day of birth. Neonatal rats were given free access to control (untreated) or drug-treated lactating rats for 14 days. Neonatal animals and lactating mothers were killed after the 2 week exposure and enzyme activities were determined in the liver homogenates as described in the Experimental section. The number of animals varied from four to nine for each treatment. Values are means $\pm$ S.D.; abbreviation used: —, not determined.

Treatment group	Catalase (units/mg of protein)	Carnitine acetyltransferase (units/mg of protein)	Peroxisomal enoyl-CoA hydratase (μ mol/min per mg)	[14 C]Palmitoyl-CoA oxidation (μ mol/min per g)
Neonates				
Control	17 $\pm$ 8	3 $\pm$ 1	1.3 $\pm$ 0.3	0.94 $\pm$ 0.11
Nafenopin	51 $\pm$ 10	108 $\pm$ 9	37.2 $\pm$ 4.0	13.32 $\pm$ 1.03
Wy-14,643	55 $\pm$ 11	41 $\pm$ 9	59.6 $\pm$ 14.9	11.11 $\pm$ 0.39
Lactating mothers				
Control	42 $\pm$ 1	—	1.0 $\pm$ 0.2	—
Wy-14,643	78 $\pm$ 7	—	48.0 $\pm$ 17.3	—

which were administered either nafenopin or Wy-14,643. The neonate liver values for enoyl-CoA hydratase reflect the maternal liver values (Tables 1) and are also consistent with reported values in adult rats exposed to hypolipidaemic drugs or the industrial plasticizer di-(2-ethylhexyl)phthalate (Lazarow, 1977; Osumi & Hashimoto, 1978).

Induction of polypeptide PPA-80 in subcellular fractions of neonate liver

The SDS/polyacrylamide gel electrophoretic patterns of large-particle and microsomal fractions from livers of control neonatal rats or from neonates whose mothers were exposed to nafenopin for 7 or 14 days are illustrated in Fig. 2. The drug treatment caused a substantial increase in the intensity of polypeptide PPA-80 in both the large-particle and

microsomal fractions of the neonate livers, as well as in the large-particle and microsomal fractions of the maternal livers (results not shown). The increased intensity of this band in the nafenopin-treated male and female neonatal rat livers correlates well with the enzymically-determined increases (29-fold, Table 1) in heat-labile peroxisomal enoyl-CoA hydratase activity (Osumi & Hashimoto, 1979), which were measured in the same large-particle and microsomal fractions. The polypeptide PPA-80 has recently been purified and shown by us to be immunochemically identical with the peroxisomal, heat-labile enoyl-CoA hydratase (Reddy, M. K. *et al.*, 1981). Polypeptide PPA-80 accounts for a large increase in peroxisomal protein in the livers of rats (M. K. Reddy *et al.*, 1980) and mice (Lalwani *et al.*, 1981) treated with peroxisome proliferators, and we have

Fig. 2. SDS/polyacrylamide slab gel electrophoretic profiles of proteins from livers of neonatal rats nursed from lactating mothers treated with the hypolipidaemic drug nafenopin

Neonatal animals were exposed to control or drug-treated lactating mothers as described in the legend to Fig. 1. Large-particle fractions (even-numbered slots) and microsomal fractions (odd-numbered slots) were prepared from neonatal rat livers and electrophoresed as described in the Experimental section. Slots 1 and 2, control neonate liver; slots 3 and 4, nafenopin-exposed neonate liver (7 day exposure); slots 5 and 6, nafenopin-exposed male neonate liver (14 day exposure); slots 7 and 8, nafenopin-exposed female neonate liver (14 day-exposure). The arrow indicates the position of the M_r 80000 polypeptide (PPA-80). Protein concentration was 20 μ g/slot.

recently demonstrated that hypolipidaemic drugs also induce polypeptide PPA-80 in mouse renal cortex (Lalwani *et al.*, 1981). In previous studies using an immunocytochemical method, the polypeptide PPA-80 was localized almost exclusively within the peroxisome matrix (Reddy *et al.*, 1981).

As a result, we can conclude that the increase of polypeptide PPA-80 (enoyl-CoA hydratase) seen on SDS/polyacrylamide gels of neonatal rat liver fractions is reflective of both peroxisome proliferation and enhanced peroxisomal β -oxidation.

Drug-induced changes in the ultrastructure of neonate and maternal livers

Neonatal rats which were exposed for 14 days to lactating mothers treated with nafenopin or Wy-14,643 displayed a marked increase in the number of peroxisomes in their liver parenchymal cells (Plates 1a and 1b). Catalase was demonstrable in all proliferated peroxisomes in the neonate livers by the cytochemical procedure (Plate 1b). The magnitude of the increase in peroxisome number in the neonate liver cells was the same as that seen in the livers of the nafenopin-treated lactating mothers (results not shown).

The results of the above experiments clearly demonstrated that the administration of hypolipidaemic drugs to the lactating mothers of suckling rats caused profound increases in the number of peroxisomes and the peroxisome-associated enzymes in neonate livers. Therefore, the peroxisome proliferating species in these lipid-lowering drugs was transferable to the neonates via maternal milk secretion. The fact that a variety of drugs and xenobiotics are capable of passing from the systemic circulation into mammalian milk is well established (Sisodia & Stowe, 1964; Darby, 1971; Rogan *et al.*, 1980). Our interest in the following experiments was to determine whether the neonates were ingesting the parent hypolipidaemic drug or a pharmacologically-active metabolite(s) via the maternal milk.

Hypolipidaemic drug transfer to suckling neonatal rats

Within 2 h after administering [3 H]nafenopin to a lactating rat by gavage, measureable levels of radioactivity were observed in milk curds isolated from the stomachs of suckling neonate rats (Fig. 3). Milk 3 H levels rose dramatically (9-fold) within the next 2 h; a concomitant rise in neonate liver 3 H content (7-fold) was also seen over this same time period (Fig. 3). A further sizeable increase in neonate liver 3 H content occurred between 4 and 6 h, concomitant with a slight drop in the milk 3 H content.

The results also indicated that radioactivity derived from either [3 H]nafenopin or [3 H]Wy-14,643 was easily measureable in neonatal rat liver homogenates after a 4 h exposure of the suckling neonates to lactating mothers which had received a gastric bolus of either one of the two radiolabelled hypolipidaemic drugs. For the above experiment, the lactating mothers were treated for 7 days with either unlabelled nafenopin or Wy-14,643 (100 mg/kg

Fig. 3. Levels of [^3H]nafenopin-derived radioactivity in neonatal rat milk curds or neonatal rat liver homogenates at specified times after administering [^3H]nafenopin to lactating rats

A lactating rat previously injected by gavage with nafenopin (100 mg/kg, twice daily for 7 days) received 100 μCi (10.1 μmol) of [^3H]nafenopin in dimethyl sulphoxide by gavage at zero time; neonates were then given free access to nurse. At specified times, neonates were sacrificed and the solid milk curds were removed from the stomach and the livers were dissected out. Curds or livers were weighed, homogenized and aliquots counted to determine [^3H] content. $\circ$ — $\circ$, milk curd; $\bullet$ — $\bullet$, liver.

body wt., twice daily) prior to the administration of radiolabelled compound. By 4 h after administering the tritiated drugs {100 μCi (10.10 μmol) of [^3H]nafenopin or 100 μCi (0.91 μmol) of [^3H]Wy-14,643} to separate mothers by gavage, similar amounts of radioactivity were measured in the neonate livers (13 000–15 000 d.p.m. of $^3\text{H/g}$ of neonate liver), indicating that an equivalent percentage of each drug had passed into the neonate circulation. This suggests that we were below the saturation point for what appears to be the simple diffusion of these drugs into milk, since there was about 11 times more mass of nafenopin administered because of its lower specific radioactivity. The ratio of neonate gastric milk curd [^3H]nafenopin: neonate liver [^3H]nafenopin content at 4 h was 42, which then decreased to 17 by 6 h, indicating a net movement of drug from the milk pool into the neonatal rat circulation (Fig. 3).

Fig. 4. T.l.c. analysis of [^3H]Wy-14,643 metabolites in organic extracts of rat milk and of liver microsomal incubations

Samples were chromatographed on silica gel plates in chloroform/acetic acid (99.9:0.1); plates were pre-developed in the running solvent and dried before the sample application. Pure [^3H]Wy-14,643 (100 μCi , 0.91 μmol) (>99.9% pure, panel a) was injected into a lactating female rat (250 g) by gastric gavage; neonates were allowed to feed from the drug-treated mother for periods up to 6 h after drug administration. At specified times (see Fig. 3) neonates were killed and gastric milk curds were removed, homogenized in 0.1 M-potassium phosphate buffer (pH 7.4) and extracted with 5 vol. of ethyl acetate. The solvent was transferred and evaporated leaving an oil; samples (5 μl) of oil were spotted on to t.l.c. plates and developed (panel b). Samples of pure [^3H]Wy-14,643 which were overlaid with 5 μl of the oil isolated from milk migrated with the same R_F as did the pure compound shown in panel (a). [^3H]Wy-14,643 (100 nmol, 2 μCi) was added to 2 ml incubations (see the Experimental section) containing 1.0 mg of liver microsomes isolated from rats (250 g) chronically fed 0.1% Wy-14,643. After a 30 min incubation at 37°C, reaction mixtures were extracted with 5 vol. of ethyl acetate. The solvent was transferred, evaporated and the residue was resuspended in 50 μl of methanol; aliquots were spotted on t.l.c. strips and developed (panel c).

T.l.c. analysis of [^3H]Wy-14,643 milk samples and [^3H]Wy-14,643 microsomal metabolites

Gastric milk curds were isolated from neonatal rats after 6 h of feeding from lactating animals which

EXPLANATION OF PLATE 1

Proliferation of peroxisomes in hepatic parenchymal cells of neonatal rats after nursing from nafenopin-treated lactating mothers for 14 days

Ultrathin sections of aldehyde-fixed, Epon-embedded liver were stained with uranyl acetate and lead citrate and examined in the electron microscope. (a), Control 14-day old neonate liver; (b), liver from neonatal rat which had nursed from a nafenopin-treated lactating mother for 14 days. The aldehyde-fixed tissue was processed for the cytochemical localization of peroxisomal catalase and then post-fixed in OsO_4 . N, nucleus; P, peroxisomes; M, mitochondria. (a), 5300 $\times$; (b), 4400 $\times$.

EXPLANATION OF PLATE 2

Hepatic peroxisome proliferation in a neonatal rat injected with purified Wy-14,643 metabolites

A 3 day old neonatal rat was injected with a pool of Wy-14,643 metabolites purified by t.l.c. from a microsomal incubation (see the Experimental section). The experimental animal (a) received subcutaneously 575 nmol of metabolites in 50 μ l of methanol/0.9% saline (1:4, v/v) once per day for 4 days; the control neonate (b) received injections of the methanol/saline carrier alone. On day 5 the animals were killed and the livers were removed and prepared for ultrastructural examination. Treatment of a neonatal rat (a) with Wy-14,643 metabolites caused a marked proliferation of electron-dense peroxisomes in liver parenchymal cells which was not observed in the liver of the control animal (b). N, nucleus; P, peroxisomes; M, mitochondria. (a), 6200 $\times$; (b), 6800 $\times$.

had received highly purified [^3H]Wy-14,643 (Fig. 4a). The homogenized milk curds were extracted with ethyl acetate, which removed 70.2% of the radioactivity from the aqueous milk phase. Control milk samples to which 1nmol of pure [^3H]Wy-14,643 was added showed greater than 99% extraction of radioactivity into ethyl acetate. Other extraction solvents which were tested (i.e. chloroform, acetone/ethyl acetate) were either equivalent or worse in their ability to extract [^3H]Wy-14,643 metabolites. Evaporation of ethyl acetate extracts of milk curd left a viscous oil; samples (5 μl) of the oil were spotted and chromatographed on silica t.l.c. plates to determine the distribution of the [^3H]Wy-14,643-derived radioactivity. Two separate peaks of radioactivity, both distributed about the origin, were seen in chromatograms of neonate milk curd extracts (Fig. 4b). When pure [^3H]Wy-14,643 was spotted on a t.l.c. plate and overlaid with the oily milk extract, normal migration of [^3H]Wy-14,643 ($R_F = 0.6$) was observed in the developed chromatogram. Thus, the non-migrating radioactivity which is seen at the origin in chromatograms of neonate milk curd extracts indicates the presence of polar [^3H]Wy-14,643 metabolites rather than the inhibited migration of [^3H]Wy-14,643 due to the oil layer. The two spots of t.l.c., combined with the unextractable material in neonate milk curds (i.e. 29.8%, see above), represent a minimum of three [^3H]Wy-14,643 metabolite fractions which are present in the milk of lactating rats, and which are transferable to neonates in the milk. There was no trace of unmetabolized [^3H]Wy-14,643 present in the neonate milk curds (Fig. 4b).

Incubation of [^3H]Wy-14,643 with microsomes derived from the livers of adult rats previously exposed to Wy-14,643 in their diet led to the quantitative conversion of the [^3H]Wy-14,643 to a product(s) which migrated on t.l.c. with an R_F (i.e. 0.1) equivalent to that seen for products found in neonate milk curd extracts (Fig. 4c). This enzymic conversion of [^3H]Wy-14,643, which was catalysed by a microsomal component, was totally abolished by prior heat denaturation of the microsomes. Again, after the 30min reaction, a significant proportion (27.6%) of the radioactivity in the microsomal incubations remained in the aqueous phase after repeated ethyl acetate extractions.

Inducing effect of Wy-14,643 metabolites

The results of the above experiments clearly showed: (a) marked peroxisome proliferation associated with increases in peroxisome enzyme levels in the livers of neonatal rats suckling from peroxisome proliferator-treated lactating mothers, and (b) milk which was consumed by the neonatal animals contained no detectable parent compound, only metabolites which showed greater polarity upon t.l.c.

analysis (Fig. 4). These results are consistent with previously observed pharmacokinetic and biotransformation data for clofibrate, a commonly used hypolipidaemic hepatic peroxisome proliferator (Thorp, 1962; Baldwin *et al.*, 1980). In the report of Baldwin *et al.* (1980), unchanged clofibrate was not detected in any of the biological samples (blood, bile, and urine) obtained from rats previously dosed with [$1\text{-}^{14}\text{C}$]clofibrate. Clofibrate is hydrolysed by plasma esterases and liver metabolizing enzymes to clofibric acid. Clofibric acid was identified as the major metabolite in plasma, whereas the glucuronide of clofibric acid was the main urinary and biliary metabolite. It would appear, therefore, that the peroxisome proliferator activity of clofibrate, Wy-14,643, nafenopin, and possibly other hypolipidaemic compounds is attributable to a metabolite(s) rather than to the parent compound. The above experimental observations led us to conclude that one (or more) of the metabolite fractions found in the milk consumed by neonates must retain the biological ability to induce the proliferation of liver peroxisomes. Whether these metabolites directly activate the genome to initiate proliferation of this organelle and induce this multienzyme system or whether they exert their action on peroxisomes indirectly by causing increased fatty acid overload in the hepatocytes remains to be elucidated.

To test directly this hypothesis, Wy-14,643 (containing tracer [^3H]Wy-14,643) was incubated with liver microsomes from Wy-14,643-treated adult rats and NADPH in a 40ml reaction mixture; the ethyl acetate extract was then preparatively chromatographed on t.l.c. as described in Fig. 4(c). The area of the chromatogram between 1.5 and 4.0cm was cut out, and the scraped silica was repeatedly extracted with ethyl acetate. The total product recovered (2300nmol, based on starting [^3H]Wy-14,643 specific radioactivity) was dissolved in methanol/0.9% saline (1:4, v/v) and injected intraperitoneally, once per day (50 μl), for 4 days, into a single neonatal rat. A littermate received equal injections of the carrier alone. The neonatal rats were fed from an untreated lactating mother rat. The electron micrographs in Plate 2 clearly indicate an increase in the number of peroxisomes in the liver of the neonate rat which was treated with the Wy-14,643 microsomal metabolite fraction.

As part of a broader aim of characterizing Wy-14,643 and nafenopin metabolism, and the pharmacological activity of their respective metabolites, we have here optimized the h.p.l.c. separation of the Wy-14,643 microsomal metabolites and have performed preliminary mass spectral analysis upon them. Two symmetric metabolite peaks were observed (Fig. 5) when organic extracts of [^3H]Wy-14,643 microsomal incubations were chromatographed on a reverse phase column

with a water/methanol gradient. From incubations containing non-radioactive Wy-14,643, each metabolite peak (I, II, Fig. 5) was collected, rotary evaporated and rechromatographed, showing the same single, u.v.-absorbing peaks. Both metabolite samples, as well as the parent compound Wy-14,643, were then examined by mass fragmentation

Fig. 5. H.p.l.c. elution profile of [^3H]Wy-14,643 and its microsomal metabolites

[^3H]Wy-14,643 ($40\ \mu\text{M}$) was incubated with rat liver microsomes ($0.8\ \text{mg/ml}$) and cofactors for 30 min. The ethyl acetate extract was evaporated and the residue was then resuspended in methanol and injected into the h.p.l.c. The compounds were resolved on a reverse phase column with a water/methanol gradient containing 0.1% acetic acid (see the Experimental section). H.p.l.c. effluent fractions were collected at 30 s intervals. Bars indicate fractions pooled from non-radioactive incubations for mass spectral analysis.

analysis on a mass spectrometer (Fig. 6). An equivalent volume of background h.p.l.c. eluate was collected and evaporated to determine background contaminant peaks in the mass spectrograms. Wy-14,643 showed molecular ions at m/z 323, 264, and 228 indicating the mass of the intact parent molecule (323), as well as the loss of the acetyl group (264) along with the chlorine atom (228), respectively (Fig. 6a). Identification of the mass fragments in the two metabolite fractions was much more ambiguous. In both cases (Figs. 6b and 6c) peaks were present at 279–280 which is consistent with a single epoxidation or hydroxylation of the Wy-14,643 ring or methyl groups, respectively (i.e. 264 plus 16). The remaining peaks seen here, which were not seen in the solvent background sample (e.g. 313, 341, 367 and 395 in Fig. 6b; 326, 368 and 394 in Fig. 6c), are significantly heavier species than one might anticipate for a single or double oxidation of the Wy-14,643 molecule. They could, however, be fragments of a coupled Wy-14,643 molecule which may have formed by the attack of an oxidized Wy-14,643 metabolite (i.e. 279) upon one of the nucleophilic nitrogen or sulphur atoms of a second Wy-14,643 metabolite. This metabolite-substrate coupling phenomenon has been previously described for the microsomal metabolism of butylated hydroxytoluene (Nakagawa *et al.*, 1979). Further analysis of these two metabolite fractions is necessary.

The studies presented here establish that the

Fig. 6. Mass spectrograms of Wy-14,643 and its microsomal metabolites

Samples of a pure Wy-14,643 stock (a) and of microsomal metabolite fractions collected from the h.p.l.c. effluent (b, c, see Fig. 5) were analysed by mass spectroscopy.

lipid-lowering drugs Wy-14,643 and nafenopin, when administered to lactating rats, can significantly increase the number of peroxisomes and the associated levels of peroxisomal enzymes in suckling rat livers. Analysis of milk samples and microsomal metabolites indicates that the peroxisome proliferative activity observed in the neonate liver is due to the transfer of a metabolite(s) in the milk. Evidence now indicates that proliferation of peroxisomes and induction of peroxisomal enzymes play a role in lipid metabolism (Reddy & Krishnakantha, 1975; Lazarow, 1978). The enhanced hepatic peroxisomal activity observed in the suckling rats, therefore, can perturb lipid metabolism in liver and possibly other tissues in these newborn animals.

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