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Increase in a specific cytochrome *P*-450 isoenzyme in the liver of congenitally jaundiced Gunn rats

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Congenitally jaundiced (*jj*) Gunn rats had a greater hepatic microsomal content of a cytochrome *P*-450 isoenzyme, *P*-450c, than did the non-jaundiced (*Jj*) rats. No differences in content of *P*-450b, *P*-450d and pregnenolone-16α-carbonitrile-induced (PCN) *P*-450 were found between *jj* and *Jj* rats. This is the first demonstration of a constitutive increase in a specific cytochrome *P*-450 isoenzyme in asociation with a genetic defect.

INTRODUCTION

Humans with the Crigler–Najjar syndrome exhibit severe, life-long unconjugated hyperbilirubinaemia, which is often fatal because of bilirubin-induced brain damage (kernicterus). The jaundiced, homozygous recessive Gunn rat is the relevant animal model of this syndrome (Schmid *et al.*, 1958). The jaundice results from a hereditary inability to form glucuronides of bilirubin, a process which normally allows its excretion into the bile. However, total bilirubin turnover is maintained at a normal rate, with an expanded pool compensating for the decreased fractional turnover of bilirubin. In the absence of conjugation, bilirubin is converted by a less efficient metabolic pathway to polar derivatives that are excreted in the bile without glucuronidation (Schmid & Hammaker, 1963). The fact that some of these derivatives are hydroxylated metabolites (Berry *et al.*, 1972; Blanckaert *et al.*, 1977) has suggested the involvement of microsomal cytochrome *P*-450-dependent monoxygenase(s) in these alternative pathways of bilirubin catabolism (Ostrow & Kapitulnik, 1986). Moreover, administration to jaundiced Gunn rats of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a potent and specific inducer of microsomal cytochrome *P*-448, produced a rapid and marked decrease in plasma bilirubin levels and total bilirubin pool (Kapitulnik & Ostrow, 1977). This treatment with TCDD accelerated the conversion of bilirubin to the same polar derivatives that were formed by the uninduced jaundiced rats, without affecting the glucuronidation of bilirubin (Kapitulnik & Ostrow, 1977). Phenobarbital (PB), which induces cytochrome *P*-450 isoenzymes different from those induced by TCDD, did not increase bilirubin catabolism in Gunn rats (Cohen *et al.*, 1985), and did not ameliorate the severe hyperbilirubinaemia of Crigler–Najjar Type I patients (Arias *et al.*, 1969). Thus, it seemed reasonable to assume that a cytochrome *P*-450 isoenzyme, which is specifically and markedly induced by TCDD, might be constitutively increased in uninduced jaundiced Gunn rats. We have therefore measured the hepatic microsomal content of several cytochrome *P*-450 isoenzymes in jaundiced and non-jaundiced newborn and adult Gunn rats.

MATERIALS AND METHODS

Microsomes were prepared (Lu & Levin, 1972) from livers of female adult (5 months old) homozygous recessive (*jj*) and heterozygous (*Jj*) Gunn rats (plasma bilirubins: 10.6–14.2 and 0.1–0.2 mg/dl, respectively), and from pooled liver of three 10 day-old *jj* and three *Jj* female littermates (plasma bilirubins: 9–16 and 0.2–0.6 mg/dl, respectively). Female adult *jj* and *Jj* Gunn rats (plasma bilirubins: 12.6 and 0.5 mg/dl, respectively) were treated with 3-methylcholanthrene (MC) (40 mg/kg in corn oil, intraperitoneally, for 2 days). Microsomes were prepared from the livers of these rats, 24 h after the last injection of MC.

Monoclonal antibodies (MAbs) specific for different forms of cytochrome *P*-450 were used to determine the microsomal content of epitope-specific cytochromes *P*-450 by a competitive radioimmunoassay (Song *et al.*, 1984). The MAbs used were specific for MC- or PB-induced rat liver cytochromes *P*-450 (Park *et al.*, 1982, 1984), and were ^{3}H-labelled by reductive methylation with NaB^3H_4. The binding of each MAb to microtitre wells, which were precoated with 2 μg of MC- or PB-induced Sprague–Dawley rat liver microsomal protein, was studied in the presence of increasing amounts of Gunn rat liver microsomes.

The cytochrome *P*-450 isoenzymes of Gunn rat liver microsomes were also analysed by the Western blot technique. Microsomes were electrophoresed on 7.5%/0.2% acrylamide/bisacrylamide gels at 20 mA,

Abbreviations used: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PB, phenobarbital; *jj*, jaundiced, homozygous recessive Gunn rat; *Jj*, non-jaundiced, heterozygous Gunn rat; MC, 3-methylcholanthrene; MAb, monoclonal antibody; AHH, arylhydrocarbon hydroxylase; PCN, pregnenolone-16α-carbonitrile.

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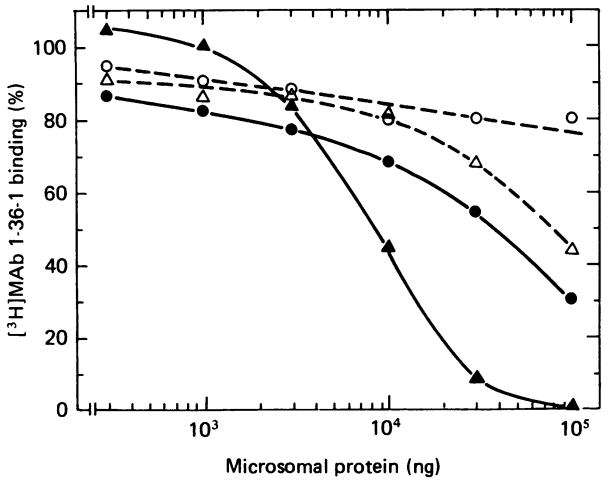

Fig. 1. Radioimmunoassay of cytochrome *P*-450c in Gunn rat liver microsomes

The binding of [^{3}H]MAb 1-36-1 to microtitre wells, each precoated with 2 μg of MC-induced rat liver microsomal protein, was studied (Song *et al.*, 1984) in the presence of increasing amounts of hepatic microsomes from female Gunn rats: △, newborn (10-day-old) non-jaundiced heterozygous (*Jj*); ▲, newborn jaundiced (*jj*); ○, adult (5-months-old) *Jj*; ●, adult *jj*. The 100% binding levels were obtained in the absence of Gunn rat liver microsomes. The data are presented as means of duplicate determinations, and differences between means were less than 5%.

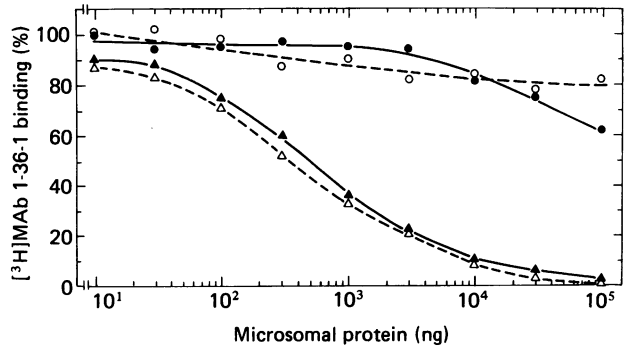

Fig. 2. Radioimmunoassay of cytochrome *P*-450c in MC-induced adult Gunn rat liver microsomes

Rats were treated with MC (40 mg/kg in corn oil, i.p.) for 2 days. The binding of [^{3}H]MAb 1-36-1 was studied, as described for Fig. 1, in the presence of increasing amounts of hepatic microsomes from adult female Gunn rats; ○, control non-jaundiced heterozygous (*Jj*); ●, control jaundiced (*jj*); △, MC-treated *Jj*; ▲, MC-treated *jj*. The data are presented as means of duplicate determinations, and differences between means were less than 5%.

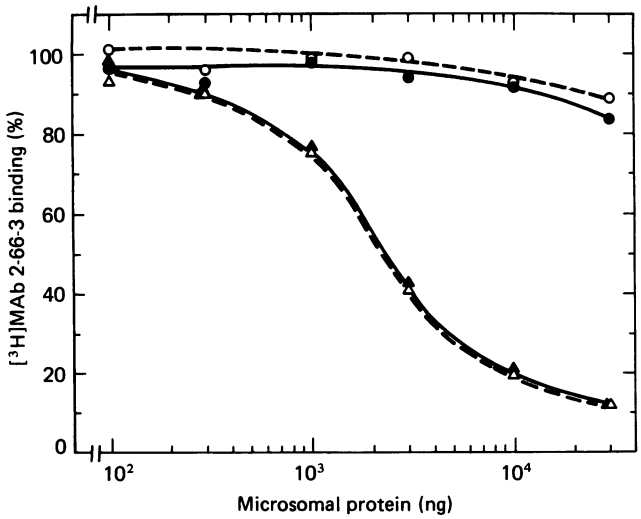

Fig. 3. Radioimmunoassay of cytochrome *P*-450b in Gunn rat liver microsomes

The binding of [^{3}H]MAb 2-66-3 to microtitre wells, each precoated with 2 μg of PB-induced rat liver microsomal protein, was studied in the presence of increasing amounts of hepatic microsomes from female Gunn rats; △, newborn non-jaundiced (*Jj*); ▲, newborn jaundiced (*jj*); ○, adult *Jj*; ●, adult *jj*. The data are presented as means of duplicate determinations, and differences between means were less than 5%.

according to the method of Laemmli (1970). Samples were then transferred to nitrocellulose sheets and treated with rabbit polyclonal antibodies to several cytochrome *P*-450 isoenzymes (Hardwick *et al.*, 1983), according to the method of Goldstein & Linko (1984). The nitrocellulose was blocked with 5% (w/v) non-fat dry milk and 0.05% Tween 20 (2 h, 37 °C), incubated with the rabbit anti-*P*-450 (5–20 μg/ml), followed by incubation with goat anti-(rabbit γ-globin) (1:200) and treatment with horseradish peroxidase–rabbit anti-peroxidase (1:2000). The blots were developed for 15 min with 4-chloro-1-naphthol (60 mg/dl) and 0.008% H_2O_2 (in 20 mM-Tris buffer, pH 8.2, containing 150 mM-NaCl). Cytochrome *P*-450 isoenzymes were quantified by densitometric scanning at 550 nm, using increasing amounts of purified isoenzyme as standard.

Arylhydrocarbon hydroxylase (AHH) activity was determined on 100 μg samples of microsomal protein, using benzo[*a*]pyrene as the substrate (Nebert & Gelboin, 1968).

RESULTS AND DISCUSSION

Liver microsomes from both newborn and adult jaundiced *jj* rats showed a greater inhibition of binding of [^{3}H]MAb 1-36-1, a MAb specific for cytochrome *P*-450c, than those from the respective non-jaundiced heterozygotes (Fig. 1). The hepatic microsomal content of cytochrome *P*-450c was almost 9-fold higher in *jj* than in *Jj* newborn animals, as shown by the respective I_{50} values (amount of protein that causes half-maximal inhibition of MAb binding): 8.6 versus 75 μg (Fig. 1). In adults, microsomal cytochrome *P*-450c was present in *jj* rats, but was not detected in the *Jj* animals (Figs. 1 and 2). Liver microsomes from *jj* rats displayed higher AHH activities than did those from *Jj* animals: 188 versus 50, and 93 versus 58, pmol of phenols formed/min per mg of protein, in newborn and adult rats, respectively. These results are consistent with the elevation of cytochrome *P*-450c, a *P*-450 isoenzyme catalytically active as an AHH.

Cytochrome *P*-450c is barely detectable in uninduced adult rat liver microsomes (Thomas *et al.*, 1983), and increases markedly upon pretreatment with polycyclic

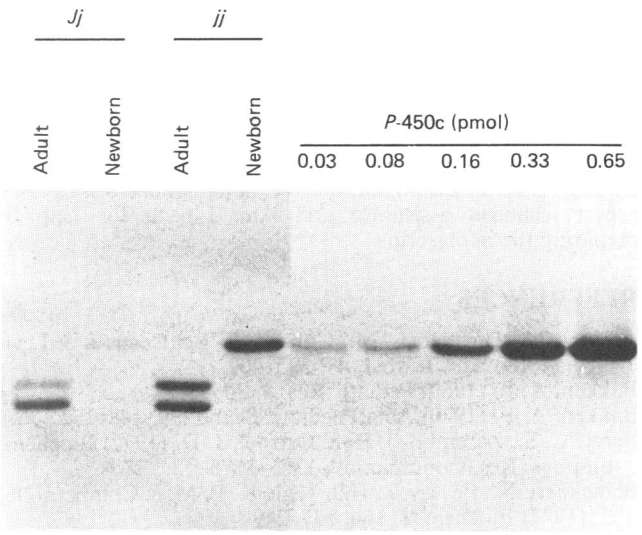

Fig. 4. Western blot analysis of cytochrome *P*-450c in Gunn rat liver microsomes

Western blot analysis with polyclonal antibodies to *P*-450 isoenzymes was performed as described in the Materials and methods section. For details of Gunn rat liver microsomal preparations see the legend to Table 1. Increasing amounts of purified *P*-450c were used as standards for quantification of this isoenzyme in the microsomal samples.

aromatic hydrocarbons such as MC and TCDD. Treatment of adult *jj* Gunn rats with MC for 2 days decreased the plasma bilirubin from 12.6 to 7.3 mg/dl, and caused major increases in the hepatic microsomal content of cytochrome *P*-450c in both *jj* and *Jj* rats (Fig. 2). Post-treatment I_{50} values for the binding of MAb 1-36-1 were similar for microsomes from jaundiced and non-jaundiced adult Gunn rats (0.5 and 0.35 μg, respectively). A similar decrease in plasma bilirubin was observed after treatment of jaundiced Gunn rats with the synthetic flavonoid 5,6-benzoflavone (J. Kapitulnik & J. D. Ostrow, unpublished work), which is another well-known inducer of cytochrome *P*-450c.

Contrary to the constitutive increase in cytochrome *P*-450c content of *jj* liver microsomes, there was no difference in cytochrome *P*-450b content between newborn *jj* and *Jj* liver microsomes, as shown by radioimmunoassay using [^{3}H]MAb 2-66-3, a MAb specific for PB-induced rat liver cytochrome *P*-450 (Park *et al.*, 1984) (Fig. 3). Cytochrome *P*-450b was not detected in liver microsomes from either *jj* or *Jj* adult Gunn rats (Fig. 3). The specific nature and extent of the constitutive increase in hepatic microsomal cytochrome *P*-450c content observed in jaundiced Gunn rats were confirmed by Western blot analysis of the microsomal samples, using polyclonal antibodies (IgGs) to several rat liver cytochrome *P*-450 isoenzymes. Thus, microsomes from uninduced *jj* rat liver had more cytochrome *P*-450c than did those from *Jj* rat liver (Fig. 4 and Table 1), and this isoenzyme was markedly induced by MC treatment (Table 1). In contrast, no major differences were found between jaundiced and non-jaundiced rats, whether newborn or adult, in the hepatic content of each of the following isoenzymes: *P*-450b, the major PB-inducible isoenzyme; *P*-450d, the major isosafrole-inducible isoenzyme; and the major pregnenolone-16α-carbonitrile (PCN)-inducible form of cytochrome *P*-450 (Table 1). In adult Gunn rat liver, the polyclonal antibody to *P*450c detected two additional bands of lower molecular mass (51.6 and 49.6 kDa) than that of cytochrome *P*-450c (55.7 kDa) (Fig. 4). These polypeptides, which were detected also in liver microsomes from uninduced adult female Sprague–Dawley rats (results not shown), were not found in newborn *jj* and *Jj* Gunn rat liver (Fig. 4). Although the 51.6 kDa polypeptide appeared to be increased in the uninduced *jj* adults (Fig. 4) it was induced only slightly after MC treatment (results not shown), in contrast with the greater than 200-fold induction of *P*-450c.

Interestingly, the liver microsomes of both *jj* and *Jj* newborn Gunn rats contained significantly higher constitutive levels of *P*-450c, *P*-450b and PCN-*P*-450 than those of the respective adult animals (Figs. 1 and 3, Table 1). Similar developmental changes have been observed in the levels of a PB-inducible cytochrome *P*-450 in Sprague–Dawley rat liver microsomes (J. Kapitulnik, unpublished work). Gonzalez *et al.* (1986) have recently reported that the levels of cytochrome *P*-450-PCN2 and its mRNA were higher in the livers of newborn (1–2 weeks old) than in those of adult female rats. Waxman *et al.* (1985) also reported age-dependent decreases in the levels of the glucocorticoid-inducible cytochrome *P*-450 isoenzyme PB-2a/PCN-E and the constitutive *P*-450 3/UT-F in female rat liver microsomes.

This is the first demonstration of a marked increase in the constitutive level of a specific cytochrome *P*-450 isoenzyme in association with a genetic defect. In *jj* Gunn rats, which either lack bilirubin:UDP glucuronyltransferase (Scragg *et al.*, 1985) or contain a defective isoenzyme (Roy Chowdhury *et al.*, 1984), plasma bilirubin levels increase rapidly soon after birth, but reach a plateau at about 2 weeks of age (Johnson &

Table 1. Western blot analysis of cytochrome *P*-450 isoenzymes in Gunn rat liver microsomes

Western blot analysis was performed, with polyclonal antibodies to the *P*-450 isoenzymes, as described in the Materials and methods section, on microsomes from: (*a*) pooled livers of three newborn (10-day-old) jaundiced homozygous (*jj*) and three non-jaundiced heterozygous (*Jj*) female littermates (20 μg of protein); (*b*) uninduced (control) adult female (5-months-old) *jj* and *Jj* Gunn rats (one rat of each; 20 μg of protein); (*c*) 3-methylcholanthrene (MC)-induced (40 mg/kg, intraperitoneal, for 2 days) adult female *jj* and *Jj* Gunn rats (one rat of each; 10 μg of protein). The cytochrome *P*-450 isoenzymes were quantified by densitometric scanning of the blots.

Rat	Iso-enzyme...	Cytochrome *P*-450 (pmol/mg of protein) c	d	b	PCN
Newborn					
jj		7.5	7.0	28	253
Jj		1.0	4.9	28	371
Adult					
jj, control		0.6	13.5	0	28
jj, MC		144			
Jj, control		0	12.1	0	27
Jj, MC		97			

Schutta, 1970; Thaler, 1970). The presence of a plateau suggests that a mechanism has developed for removal of bilirubin, concordant with the marked perinatal increase which we observed in hepatic cytochrome *P*-450c content. The rapid postnatal increase in serum bilirubin levels in the jaundiced newborn Gunn rats may thus be an endogenous trigger for the marked increase in cytochrome *P*-450c content of hepatic microsomes in these animals. Interestingly, it has been suggested that hyperbilirubinaemia may play a role in the perinatal development of bilirubin:UDP glucuronyltransferase. This assumption was derived from the presence of conjugated bilirubin in amniotic fluid (Jansen *et al.*, 1969) as well as in cord blood serum of severely jaundiced infants with erythroblastosis fetalis and newborn babies born to jaundiced mothers (Bakken, 1970). Newborn *Jj* Gunn rats delivered from jaundiced females had higher bilirubin:UDP glucuronyltransferase activities at birth than did the heterozygous offspring of non-jaundiced mothers (Thaler, 1970). However, disparate results were obtained when the hepatic glucuronidation of bilirubin was measured in newborn Wistar rats whose mothers were infused with bilirubin during the last week of pregnancy (Bakken, 1969; Winsnes & Bratlid, 1973).

The increased constitutive levels of hepatic cytochrome *P*-450c in jaundiced Gunn rats, as well as the augmented formation and biliary excretion of hydroxylated bilirubin derivatives after pretreatment with TCDD (Kapitulnik & Ostrow, 1977), a potent inducer of cytochrome *P*-450c, suggest a role for this cytochrome *P*-450 isoenzyme in the maintenance of a normal total bilirubin turnover in these animals and in humans with the comparable Crigler–Najjar syndrome. In contrast, the lack of a difference in cytochrome *P*-450b content between *jj* and *Jj* liver microsomes and the failure of phenobarbital to increase bilirubin catabolism in jaundiced Gunn rats (Cohen *et al.*, 1985) or in patients with Type I Crigler–Najjar syndrome (Arias *et al.*, 1969) suggest that cytochrome *P*-450b is not involved in the alternative pathways of bilirubin catabolism that develop when glucuronidation is deficient. The mitochondrial bilirubin oxidase described by Cardenas-Vazquez *et al.* (1986) has K_m and $V_{max.}$ values that seem appropriate to the concentrations *in vivo* and metabolic rates for bilirubin in the Gunn rat. However, the activity of that oxidase was only insignificantly higher in the jaundiced, as compared with the non-jaundiced, rats and was not increased by β-naphthoflavone, which has been shown to accelerate the catabolism of bilirubin in jaundiced Gunn rats. Thus, this mitochondrial oxidase cannot account for the enhancement of the alternative bilirubin metabolic pathways(s) by TCDD and related inducers, and its role *in vivo* in bilirubin metabolism in jaundiced animals remains to be established. Further understanding of the endogenous acceleration of bilirubin catabolism in jaundiced Gunn rats, and the ultimate development of a specific inducer of cytochrome *P*-450c devoid of the toxic properties of polycyclic aromatic hydrocarbons, could be of great value in improving the management of severe hereditary unconjugated hyperbilirubinaemia.

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REFERENCES

Arias, I. M., Gartner, L. M., Cohen, M., BenEzzer, J. & Levi, A. J. (1969) Am. J. Med. **47**, 395–409.
Bakken, A. F. (1969) Pediatr. Res. **3**, 205–209
Bakken, A. F. (1970) Acta Paediatr. Scand. **59**, 148–152
Berry, C. S., Zarembo, J. E. & Ostrow, J. D. (1972) Biochem. Biophys. Res. Commun. **49**, 1366–1375
Blanckaert, N., Fevery, J., Heirwegh, K. P. M. & Compernolle, F. (1977) Biochem. J. **164**, 237–249
Cardenas-Vazquez, R., Yokosuka, O. & Billing, B. H. (1986) Biochem. J. **236**, 625–633
Cohen, A. N., Kapitulnik, J., Ostrow, J. D., Cochrane, C. & Celic, L. (1985) Hepatology **5**, 310–316
Goldstein, J. A. & Linko, P. (1984) Mol. Pharmacol. **25**, 185–191
Gonzalez, F. J., Song, B.-J. & Hardwick, J. P. (1986) Mol. Cell. Biol. **6**, 2969–2976
Hardwick, J. P., Gonzalez, F. J. & Kasper, C. B. (1983) J. Biol. Chem. **258**, 10182–10186
Jansen, F. H., Heirwegh, K. P. M. & Devriendt, A. (1969) Lancet **i**, 702–704
Johnson, L. & Schutta, H. S. (1970) in Bilirubin Metabolism in the Newborn (Bergsma, D. & Hsia, D. Y. Y., eds.), pp. 114–118, Williams and Wilkins, Baltimore
Kapitulnik, J. & Ostrow, J. D. (1977) Proc. Natl. Acad. Sci. U.S.A. **75**, 682–685
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lu, A. Y. H. & Levin, W. (1972) Biochem. Biophys. Res. Commun. **46**, 1334–1339
Nebert, D. W. & Gelboin, H. V. (1968) J. Biol. Chem. **243**, 6242–6249
Ostrow, J. D. & Kapitulnik, J. (1986) in Bile Pigments and Jaundice: Molecular, Metabolic, and Medical Aspects (Ostrow, J. D., ed.), pp. 421–438, Marcel Dekker, New York
Park, S. S., Fujino, T., West, D., Guengerich, F. P. & Gelboin, H. V. (1982) Cancer Res. **42**, 1798–1808
Park, S. S., Fujino, T., Miller, H., Guengerich, F. P. & Gelboin, H. V. (1984) Biochem. Pharmacol. **33**, 2071–2081
Roy Chowdhury, J., Roy Chowdhury, N. & Arias, I. M. (1984) Biochem. Soc. Trans. **12**, 81–83
Schmid, R. & Hammaker, L. (1963) J. Clin. Invest. **42**, 1720–1734
Schmid, R., Axelrod, J., Hammaker, L. & Swarm, R. L. (1958) J. Clin. Invest. **37**, 1123–1130
Scragg, I., Celier, C. & Burchell, B. (1985) FEBS Lett. **183**, 37–42
Song, B.-J., Fujino, T., Park, S. S., Friedman, F. K. & Gelboin, H. V. (1984) J. Biol. Chem. **259**, 1394–1397
Thaler, M. M. (1970) Science **170**, 555–556
Thomas, P. E., Reik, L. M., Ryan, D. E. & Levin, W. (1983) J. Biol. Chem. **258**, 4590–4598
Waxman, D. J., Dannan, G. A. & Guengerich, F. P. (1985) Biochemistry **24**, 4409–4417
Winsnes, A. & Bratlid, D. (1973) Biol. Neonate **22**, 367–373

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