Perinatal Development of, and Effect of Chemical Pretreatment on, Glycine *N*-Acyltransferase Activities in Liver and Kidney of Rabbit and Rat

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The ontogenic development of glycine *N*-acyltransferase activity was studied in preparations of hepatic and renal mitochondria from the New Zealand White rabbit and the Sprague–Dawley rat. Preparations of hepatic mitochondria from the rat and the rabbit attain adult glycine *N*-acyltransferase specific activities by birth and 4 weeks of age respectively, whereas mitochondrial preparations from rabbit kidney do not attain adult activity until 4 months of age. Pretreatment of adult rats or immature rabbits with salicylic acid, benzoic acid or phenobarbital had little effect on glycine *N*-acyltransferase activity *in vitro* in liver or kidney.

In most mammals, kidney and liver are able to catalyse conjugation of carboxylic acids with amino acids. Examples of carboxylic acids that undergo the reaction *in vivo* are salicylic acid (Wan & Riegelman, 1972), 2-(2,4-dichlorophenoxy)phenylacetic acid (Jordan & Rance, 1974) and other carboxylic acid drugs or metabolites of drugs (Lan *et al.*, 1975; Drach & Howell, 1968), benzoic acid (Bridges *et al.*, 1970), phenylacetic acid (James *et al.*, 1972) and other carboxylic acids from dietary sources (see Petterson & Jellum, 1972; Gompertz & Draffan, 1972; Ando *et al.*, 1973; Solheim & Scheline, 1973; Strand & Scheline, 1975), certain herbicides (Grunow & Bohme, 1974; James & Bend, 1976), pesticides (Kamienski & Casida, 1970) and solvents (Flek & Sedivec, 1976; Hutson & Pickering, 1971) and their metabolites. The last three classes of carboxylic acids are commonly encountered in the environment.

Their rates of conjugation may be expected to affect markedly the half-life periods of these acids in the body. In early life, many conjugating pathways have lower activities than are normal in adults (Bend *et al.*, 1975; James *et al.*, 1977) and in newborn animals this can affect the toxicity of substances that have to be conjugated before excretion.

We have developed an assay for measuring formation of phenylacetyl-glycine from phenylacetyl-CoA and glycine (James & Bend, 1978) and have used it to study the developmental pattern of glycine *N*-acyltransferase in rabbit liver and kidney and rat liver. Brandt (1966) described the development of glycine *N*-acyltransferase in rat liver, but the assay used measured the disappearance of substrate, and the development of activity in kidney, the major site of conjugation in many mammals (Von Lehmann *et al.*, 1973), was not reported.

We also examined the effect of pretreating young rabbits and adult rats with sodium salicylate to see whether this would induce conjugating activity, since one report (Irrjala, 1972) has shown that treatment of rats with salicylate increased the rate of formation *in vitro* of 4-aminohippurate from 4-aminobenzoate and of salicylurate from salicylate. In another study, Garretson *et al.* (1975) suggested that a female infant born to a woman who ingested 6.5 g of aspirin daily throughout pregnancy was better able to excrete salicylate as salicyluric acid than infants whose mothers took single doses of aspirin just before giving birth. In other attempts to induce glycine *N*-acyltransferase, we administered sodium phenobarbital or sodium benzoate to rats. In rats, phenobarbital causes an increase in liver weight associated with an increase in synthesis of some proteins, especially enzymes that metabolize foreign chemicals (Conney, 1967). Phenobarbital has been shown to induce at least two families of conjugating enzymes, the cytosol fraction glutathione *S*-transferases (Ryan *et al.*, 1976) and the microsomal UDP-glucuronyltransferases (Vaino, 1973).

A preliminary report of this study was presented at the 59th Annual Meeting of the Federation of American Societies for Experimental Biology and Medicine at Atlantic City (James & Bend, 1975).

Experimental

Materials

Chemicals and equipment used in this study were as described in the preceding paper (James & Bend, 1978).

Animals

The rabbits were of the New Zealand White strain and were purchased from Franklin's Rabbitry, Wake Forest, NC, U.S.A. They were artificially inseminated after injection of pituitary lutropin (luteinizing hormone) to cause ovulation, and the day of insemination was taken as day zero of gestation. Three pregnant females were each killed on gestational days 17, 20, 25, 28 and 30, and litters at several different times after birth. Three to four litters at each age were studied. The livers and kidneys, and where appropriate, placenta, were removed from each animal. At gestational days 17–25, only placenta was studied for glycine *N*-acyltransferase activity. At gestational days 28 and 30, and up to 2 weeks of age, the livers and kidneys were pooled from each litter (average size six pups). Individual organs were assayed separately from young rabbits aged 4–15 weeks. No attempt was made to distinguish between sexes in the offspring, since no sex difference was found in previous studies with adult rabbits (see preceding paper). Activities were assayed in four adult female rabbits (3.2–4.0 kg; 6–10 months) and in four adult males (3.0–3.3 kg; 6–8 months) as references.

Rats used to study the perinatal development of glycine *N*-acyltransferase were date-bred Sprague-Dawley rats (CD strain) purchased from Charles River Laboratories, Wilmington, MA, U.S.A. Livers were pooled from several litters at each time point between 17 days gestation and 20 days of age. The adult references were seven non-pregnant females (10 weeks old, 220–280 g) and four males (8 weeks old, 215–250 g). The adult animals used to study the effects of salicylate and phenobarbital were Sprague-Dawley (CD) rats bred at National Institute of Environmental Health Sciences (NIEHS). Rats used to study the effect of benzoic acid on glycine *N*-acyltransferase activity were purchased from Sprague-Dawley, Madison, WI, U.S.A.

Tissue preparation

For the study of the development of glycine *N*-acyltransferase activity with age, rat hepatic mitochondria were prepared as described by Woods (1975) and resuspended in cold (0–4°C) 0.02 M-Tris/HCl, pH 7.6, so that the final protein concentration was 10–20 mg/ml. Triton X-100 was included in the incubation tube when glycine *N*-acyltransferase was assayed.

In other studies with rats, preparations of hepatic and renal subcellular fractions from individuals were prepared as described for rabbits, except that 9 g of liver and both kidneys (about 2.5 g) were used.

Where animal or pool size permitted, we prepared subcellular fractions from 18 g of rabbit liver and placenta and 9 g of rabbit kidney, using the conditions described in the preceding paper (James & Bend,

1978). Mitochondrial suspensions, and Triton X-100-solubilized preparations from mitochondrial suspensions were used in assays. In some experiments, suspensions of the 600g pellets, and Triton-X-100-solubilized preparations of the 600g pellet were also assayed. The 13 300g supernatant fractions and samples of whole homogenized suspension treated with 1% Triton X-100 (2:1, v/v) were sometimes assayed for activity in experiments where rats had been pretreated with chemicals.

Assays

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin fraction V (Metrix, Chicago, IL, U.S.A.) as standard.

Glycine *N*-acyltransferase was assayed by the radiochemical method of James & Bend (1978), with phenylacetyl-CoA as the acyl acceptor.

Succinate-cytochrome *c* reductase is widely accepted as a marker enzyme for inner mitochondrial membranes (de Duve *et al.*, 1955) and was as a routine assayed by the method of Yamashita & Racker (1969) to ensure that mitochondria were concentrated in the 13 300g pellet by our centrifugation procedure, since subcellular fractions prepared from suspensions of organs from immature animals may be precipitated at different speeds from suspensions of the same organs from adult animals.

Pretreatment with chemicals

In all experiments where animals were pretreated with chemicals, glycine *N*-acyltransferase activity was assayed in Triton X-100-solubilized preparations of renal and hepatic mitochondria. In some experiments, other fractions were also assayed (see under 'Tissue preparation').

Salicylate

The ten rabbits used in the salicylate-pretreatment study were litter mates. Five were dosed orally with aq. sodium salicylate (350 mg/kg) for 4 days, and five at the same time with an equal volume of water. All animals were killed 24 h after the last dose. Their age was then 8 weeks.

The same schedule was used to treat seven male and four female rats with sodium salicylate (350 mg/kg). In addition, seven female rats were dosed for only 3 days and killed at 24 h (four rats) or 48 h (three rats) after the last dose, and four of the seven male rats treated for 4 days were not killed until 48 h after the last dose. Four female and four male rats treated with 0.9% NaCl for 4 days and then killed after 24 h served as controls.

Benzoate

Two routes of administration were used, oral and

intraperitoneal. Four male rats were given free access to aq. 10mM-sodium benzoate instead of water, for 34 days before death, and four male rats given free access to water served as controls. Intraperitoneal doses of sodium benzoate (340mg/kg) were given to five male and five female rats for 4 days, and five control rats of each sex received injections of 0.9% NaCl. Rats were killed 48h after the last dose.

Phenobarbital

Four female rats were injected intraperitoneally with a solution of sodium phenobarbital (80mg/kg) for 3 days, and another group of three female rats with an equal volume of 0.9% NaCl for 3 days. The rats were killed on day 4.

Statistical analysis

Pairwise comparisons of means were made by Mann-Whitney U tests (Hollander & Wolfe, 1973). *P* values of less than 0.05 were taken to represent significant differences between means.

Results

Ontogeny of glycine *N*-acyltransferase in rabbits

The centrifugation conditions used led to some sedimentation of mitochondria with the nuclei, as judged by the presence of succinate-cytochrome *c* reductase and glycine *N*-acyltransferase activities in the 600g pellet, especially in the liver of rabbits below 28 days of age. However, the 13 300g or mitochondrial pellet also always contained both glycine *N*-acyltransferase and succinate-cytochrome *c* reductase activities. Activity in the mitochondria was used to investigate the age-related development of glycine *N*-acyltransferase, since we have evidence to suggest that glycine *N*-acyltransferase is located in the matrix of the mitochondrion in liver and kidney from adult rabbits (see preceding paper). Enzyme activities in the hepatic 600g pellets and Triton-solubilized preparations of the 600g pellet were monitored at a few time points and increased with age in parallel with hepatic mitochondrial preparations.

Very low transferase activities (less than 0.01 nmol/min per mg of protein) were found in placenta at gestational ages 17, 20, 25, 28 or 30 days. However, placental mitochondria were very active in hydrolysing phenylacetyl-CoA to phenylacetic acid.

Before 28 days of gestation, foetal kidneys were very small, so foetal liver and kidneys were only assayed from 28 days onward. The gestational period in rabbits is normally 31 days.

Fig. 1 shows the development of glycine *N*-acyltransferase activity in kidney and liver Triton-solubilized preparations of mitochondria. It should be noted that adult specific activity in liver was much less than in kidney. Mean specific activity in liver from adult male rabbits tended to be lower than that in

Fig. 1. Ontogeny of glycine *N*-acyltransferase specific activity in mitochondrial preparations from liver and kidney of New Zealand White rabbits

●, Mean specific glycine *N*-acyltransferase activity at each age in Triton X-100-solubilized preparations of renal mitochondrial fractions; ○, mean specific glycine *N*-acyltransferase activity at each age in Triton-solubilized preparations of hepatic mitochondrial fractions. Activities are means $\pm$ s.d. ($n = 3$) for pools of liver and kidney as described in the text.

liver from female rabbits, but the variability was high, especially in males, and the difference was not statistically significant.

In Triton X-100-solubilized mitochondrial preparations and also in Triton X-100-solubilized 600g pellet from rabbit liver, adult glycine *N*-acyltransferase activities were found from 4 weeks of age onward, whereas in renal mitochondrial preparations, activities did not attain adult male values until 12 weeks of age and were below the adult range of female specific activities even at 12 and 15 weeks of age.

The total organ activity of glycine *N*-acyltransferase calculated from the specific activity in Triton-solubilized preparations of mitochondria and the total yield of mitochondria per organ showed that rabbit kidney mitochondria have about five times the total activity of rabbit liver mitochondria. The total activity per organ calculated for both kidneys was $14.2 \pm 1.2 \mu\text{mol/min}$ for adult females, and $16.3 \pm 6.6 \mu\text{mol/min}$ for adult males (means $\pm$ s.d., $n = 4$),

Fig. 2. Ontogeny of glycine *N*-acyltransferase specific activity in rat hepatic mitochondria treated with Triton X-100

Livers were pooled from 20–30 young rats (three litters). Adult activities shown are means $\pm$ S.D., n = five individuals.

whereas for whole liver it was 3.5 ± 1.3 and 2.1 ± 2.2 $\mu\text{mol/min}$ (means $\pm$ S.D., n = 4 for females and males respectively). These values do not represent the total capacity of liver and kidney to catalyse glycine conjugation, since some mitochondria were present in the 600g pellet. Nevertheless, data similarly obtained show that, under our experimental conditions, total organ activity increased gradually with age in liver and kidney, and the apparent increase above adult activities in Triton-solubilized preparations from liver was not seen when total organ activities of the mitochondrial fractions were compared.

Ontogeny of glycine *N*-acyltransferase in rats

The perinatal development of glycine *N*-acyltransferase activity in rat liver mitochondria assayed in the presence of Triton X-100 is shown in Fig. 2. The gestational period for rats is about 21 days. Near-adult

Table 1. Effect of chemicals on glycine *N*-acyltransferase activity in rats

Rats were treated with sodium salicylate, sodium benzoate, sodium phenobarbital, 0.9% NaCl or water as described in the Experimental section. At the stated times, the rats were killed, and livers and kidneys removed. Triton-solubilized preparations of mitochondria were made from the organs of individual rats and used to assay glycine *N*-acyltransferase activities. Activities shown are means $\pm$ S.D. for the numbers of individuals indicated in parentheses.

Treatment	Route	Sex	Activity (nmol conjugated/min per mg of protein)	
			Liver	Kidney
Sodium salicylate (10)	Oral	Female	20.7 $\pm$ 4.8*	34.0 $\pm$ 9.6*
0.9% NaCl (control) (4)	Oral	Female	13.3 $\pm$ 0.8	23.5 $\pm$ 5.3
Sodium salicylate (7)	Oral	Male	16.5 $\pm$ 2.6*	44.2 $\pm$ 9.3*
0.9% NaCl (control) (4)	Oral	Male	10.5 $\pm$ 3.8	29.0 $\pm$ 8.8
Sodium benzoate (4)	Oral	Male	34.3 $\pm$ 3.9	71.7 $\pm$ 5.9*
Water (control) (4)	Oral	Male	32.6 $\pm$ 9.7	59.5 $\pm$ 6.7
Sodium benzoate (5)	Intraperitoneal	Female	35.0 $\pm$ 9.8	69.7 $\pm$ 7.6†
0.9% NaCl (control) (5)	Intraperitoneal	Female	37.0 $\pm$ 5.4	51.0 $\pm$ 2.5
Sodium benzoate (6)	Intraperitoneal	Male	32.7 $\pm$ 7.8	63.5 $\pm$ 24.5
0.9% NaCl (control) (4)	Intraperitoneal	Male	32.3 $\pm$ 4.5	54.5 $\pm$ 2.6
Sodium phenobarbital (4)	Intraperitoneal	Female	30.5 $\pm$ 1.1	36.5 $\pm$ 4.1*
0.9% NaCl (control) (3)	Intraperitoneal	Female	23.2 $\pm$ 6.7	23.3 $\pm$ 2.9

* P < 0.05, treated versus control.

† P < 0.01, treated versus control.

specific enzyme activity was present at birth, relative to adult female activity, and by gestational day 19 relative to adult males. As with rabbits, total organ activities for livers from rats aged 4–16 days were lower than for adult rats.

Pretreatment with chemicals

Rats purchased from Sprague–Dawley, and used to study the effects of sodium benzoate administration, had consistently higher hepatic and renal glycine *N*-acyltransferase specific activities than rats bred at NIEHS, which were used to study the effect of salicylate or phenobarbital pretreatment.

Salicylate. Treatment of young rabbits with salicylate for 4 days did not alter glycine *N*-acyltransferase activity measured in Triton-solubilized mitochondria, with respect to untreated controls. Specific activity in Triton-solubilized preparations of liver mitochondrial fractions was 4.9 ± 3.1 and 3.7 ± 1.6 nmol conjugated/min per mg of protein in control and treated animals respectively (means $\pm$ S.D., $n = 5$). Specific activities in Triton-solubilized preparations of kidney mitochondrial fractions were 44.4 ± 13.4 and 44.9 ± 6.1 nmol conjugated/min per mg of protein for control and treated rabbits respectively (means $\pm$ S.D., $n = 5$). There were no significant differences in body weight or liver or kidney weight between the groups, and total organ activities were in the same range.

Male and female rats given oral doses of sodium salicylate had significantly higher activities of glycine *N*-acyltransferase in Triton-solubilized preparations of liver and kidney mitochondria (Table 1). Salicylate treatment did not affect the liver or kidney weights of these rats. The same range of activities was observed when rats were killed 24 or 48 h after the last dose, so all results for treated animals were pooled. This was also true for the benzoate-treated rats.

Benzoate. Intraperitoneal injection of sodium benzoate significantly increased the activity of glycine *N*-acyltransferase in solubilized preparations of kidney mitochondria from treated female rats above controls, but did not affect liver activity (Table 1). In male rats there was no difference between treated and control activities in the liver or kidney. In both treated and control rats, about 30% of the total kidney activity found in Triton-treated whole-kidney homogenized suspensions could be accounted for in Triton-solubilized preparations of kidney mitochondrial fractions. The value for liver was 10%.

Administration of sodium benzoate to male rats in their drinking water did not affect hepatic glycine *N*-acyltransferase, but caused a slight increase in renal mitochondrial activities. Activities in renal cytosol fractions were the same for treated and control rats.

Phenobarbital. Pretreatment of female rats (220–

240 g) with phenobarbital increased glycine *N*-acyltransferase activity in Triton-solubilized preparations of mitochondria from both liver and kidney (Table 1). However, only in kidney preparations was the increase statistically significant. Kidney weight and mitochondrial protein yield were unaffected by phenobarbital pretreatment, whereas liver weight, but not mitochondrial yield, increased in rats that had received phenobarbital.

Discussion

Specific and total glycine *N*-acyltransferase activities in liver and kidney preparations from foetal and newborn rabbits were low compared with adult activities and rose slowly postnatally, whereas in rat liver, adult activities of glycine *N*-acyltransferase were present by birth. Other conjugating enzyme activities, including glutathione *S*-transferase activities toward 1,2-dichloro-4-nitrobenzene, and styrene oxide, follow a similar slow developmental profile in liver and kidney of rabbit (James *et al.*, 1977). Our finding that placenta had high acyl-CoA hydrolase activity, but no conjugating activity, at least from gestational age 17 days, suggests that it is not a site for amino acid conjugation of carboxylic acids.

In rabbit and other species where conjugating activity matures slowly, compounds that are conjugated before elimination may be toxic to the foetus and newborn animal at doses that are not toxic to the normal adult.

Toxic effects of exogenous carboxylic acids should be more easily elicited under conditions of chronic exposure, unless this induces glycine *N*-acyltransferase activity or causes increases in the availability of glycine for conjugation. This may be important in cases where infants are exposed to high concentrations of carboxylic acids, as in phenylketonuria and other metabolic acidemia conditions, or where their mothers have ingested salicylate drugs or benzoate from soft drinks. We found that oral administration of salicylic acid to young rabbits did not increase the specific activity of glycine *N*-acyltransferase in liver or kidney. We have no information as to how carboxylic acids affect glycine concentration in the matrix of the mitochondrion, but we postulate that unless glycine concentration increases, young rabbits will be intoxicated by carboxylic acids at lower doses than adults.

Benzoic acid and phenylacetic acid are both produced endogenously in adult animals, including man, by bacterial metabolism in the intestine, and are absorbed, then excreted in urine as conjugates (Seakins, 1971). Thus non-nutritive carboxylic acids which are products of normal dietary intake, as well as those from other environmental origins, utilize the amino acid conjugation pathway to facilitate their excretion. It is possible, but has not been demon-

strated, that the rate of development of the factors controlling bacterial production of benzoic acid and phenylacetic acid in rabbit intestine parallels the development of factors controlling the amount of available glycine and the maturation in rabbit kidney of glycine *N*-acyltransferase.

Renal and hepatic preparations from adult male or female rats treated with salicylate had about 1.5 times the specific glycine *N*-acyltransferase activities of control animals. Irjala (1972), who assayed the complete reaction in rat liver and kidney slices, found a similar overall increase, between 1.5- and 2-fold, in the synthesis of salicylglycine from salicylic acid and of 4-aminobenzoylglycine from 4-aminobenzoic acid.

Renal glycine *N*-acyltransferase activity was only slightly increased (about 1.3-fold) or was unaffected by repeated administration of benzoic acid to male or female rats. Liver enzyme activity was not increased. Phenobarbital pretreatment, which causes induction of a wide variety of enzymes, gave a more marked increase (1.6-fold) in acyltransferase activity of rat kidney, but did not increase hepatic specific activity significantly.

Conjugation of benzoic acid and phenylacetic acid with glycine *in vivo* increases with exogenous doses of either acid, especially if glycine is administered concomitantly (Wan & Riegelman, 1972; Amsel & Levy, 1970). At very high doses of benzoic acid, if glycine is not also administered, excretion of hippurate reaches a plateau; that is, the rate of glycine conjugation is limited. However, the rate of excretion of salicylglycine is constant regardless of increasing doses of salicylic acid or concomitant administration of glycine (Levy, 1971). From these data and other pharmacological evidence, Levy concluded that the rate of excretion of salicylic acid is probably limited by the rate of its activation to salicyl-CoA, whereas the rate of excretion of benzoic acid is probably limited by the availability of glycine for the reaction of benzoyl-CoA with glycine. Other evidence supporting the theory that the concentration of glycine limits the rate of conjugation of phenylacetic acid and benzoic acid comes from data obtained by assays *in vitro* of glycine *N*-acyltransferase activity. The apparent K_m for glycine in renal preparations from rabbit was 20 mM (James & Bend, 1978), and in renal preparations from rat, 15 mM (Schachter & Taggart, 1954). These K_m values suggest that high concentrations of glycine are necessary for maximum conjugating activity. The variation in K_m and V_{max} values for the formation of the different acyl-CoA substrates of glycine *N*-acyltransferase may therefore account for the differences observed *in vivo* in the conjugation of various carboxylic acids.

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