Identification of increased amounts of UDP-glucuronyltransferase protein in phenobarbital-treated chick-embryo liver cells

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1. UDP-glucuronyltransferase activity of neonatal-chick liver or phenobarbital-treated chick-embryo liver catalysed the glucuronidation of 1-naphthol, 4-nitrophenol and 2-aminophenol. Only low transferase activity towards testosterone was detected, and activity towards bilirubin was not detectable. 2. Liver microsomal transferase activity towards the three phenols was increased approx. 20–50-fold by phenobarbital treatment of chick embryos or by transfer of liver cells into tissue culture. 3. A single form of UDP-glucuronyltransferase, which appears to catalyse the glucuronidation of these three phenols, was purified to near homogeneity from phenobarbital-treated chick-embryo liver microsomal fraction for the first time. 4. The use of this purified enzyme as a standard protein facilitated the identification of this protein in chick-embryo liver microsomal fraction. Further, the accumulation of this microsomal protein was observed following phenobarbital treatment of chick embryos and during tissue culture of chick-embryo liver cells. 5. The value of this model system for the study of the induction of UDP-glucuronyltransferase by drugs and hormones is discussed.

The molecular mechanisms involved in the regulation of drug-metabolizing enzymes by drugs and hormones are currently the subject of vigorous investigation by a number of laboratories. These investigations in mammalian systems are complicated by the heterogeneity of drug-metabolizing enzymes and the specificity or magnitude of measurable changes in response to drug administration (see Nebert & Negishi, 1982). Further, the molecular mechanisms responsible for the natural development of these enzymes perinatally remain obscure (Dutton, 1980).

The developing chick embryo is in many respects a more convenient model for tackling some of these problems than is the mammalian system (see Dutton *et al.*, 1976). Glucuronidation, a major route of conjugation of toxic endogenous and xenobiotic molecules, is virtually absent from chick-embryo liver until hatching, when the activity of the rate-limiting enzyme UDP-glucuronyltransferase (EC 2.4.1.17) rises suddenly to adult values (Dutton & Ko, 1966). Precocious development *in ovo* of this enzyme can be brought about by injection of phenobarbital or infusion of certain 11β -hydroxy-corticosteroids into embryonated eggs (Wishart & Dutton, 1975; Leakey *et al.*, 1976) such that

UDP-glucuronyltransferase activity is increased from virtually zero by some 50-fold by corticosterone acetate (Leakey *et al.*, 1976) and by up to 200-fold by phenobarbital (Wishart & Dutton, 1975). Further, the heterogeneity of UDP-glucuronyltransferase, well recognized in mammalian liver (Burchell, 1981), is not obvious and not yet recognized in chick liver (Dutton, 1980). These enormous increases in UDP-glucuronyltransferase activity mediated by phenobarbital in chick-embryo liver *in ovo* can also be demonstrated in chick-embryo liver cultures (Burchell *et al.*, 1972). Thus the developing chick-embryo liver cell provides an excellent model for study of the molecular mechanisms involved in regulation of UDP-glucuronyltransferase activity by drugs and hormones.

Earlier studies using chick-embryo liver cultures indicated the requirement for protein synthesis during the appearance of UDP-glucuronyltransferase activity in response to phenobarbital (Burchell *et al.*, 1972). However, we cannot begin to study the transcriptional or translational events of this induction process without first being able to recognize an individual gene product, which will then facilitate the study of the genetic regulation of UDP-glucuronyltransferase.

In this paper we describe a method for purification of a chick-embryo liver UDP-glucuronyl-

Abbreviation used: SDS, sodium dodecyl sulphate.

transferase for the first time, and we are able to observe the phenobarbital-stimulated increase in the amount of this microsomal protein *in ovo* and the spontaneous appearance of UDP-glucuronyltransferase protein in monolayer hepatocyte cultures.

Experimental

Materials

Lubrol 12A9 (a condensate of dodecyl alcohol with approx. 9.5 mol of ethylene oxide/mol) was obtained from ICI Organics Division, Manchester, U.K. UDP-glucuronic acid (triammonium salt), bilirubin, 4-nitrophenol, bovine serum albumin, corticosterone acetate and Coomassie Brilliant Blue protein stain were all purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. The radioactive materials 1-[1-¹⁴C]naphthol and [4-¹⁴C]testosterone were from The Radiochemical Centre, Amersham, Bucks., U.K. 2-Aminophenol (twice resublimed before use) and sodium phenobarbital were from BDH Chemicals, Poole, Dorset, U.K. All other chemicals, where available, were analytical-reagent grade. Egg phosphatidylcholine (grade 1) was purchased from Lipid Products, South Nutfield, Surrey, U.K.

The protein standards used for SDS/polyacrylamide-gel electrophoresis (bovine serum albumin, pyruvate kinase, fumarase and aldolase) were purchased from Boehringer, Mannheim, West Germany.

CNBr-activated Sepharose CL-4B was from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose (DE 52) was obtained from Whatman Biochemicals, Maidstone, Kent, U.K.

Fertile eggs, from a 'synthetic' cross-bred female line (broilers), were from Ross Poultry Farm, Colliston, nr. Arbroath, Scotland, and were incubated in an egg incubator at 37–38°C and 55–60% relative humidity. Embryo age was dated from the start of incubation.

Enzyme assays

Hepatic microsomal UDP-glucuronyltransferase activity towards various substrates was assayed by the following methods: bilirubin (Heirwegh *et al.*, 1972), 1-naphthol (Otani *et al.*, 1976), testosterone (Rao *et al.*, 1976), 2-aminophenol and 4-nitrophenol (Winsnes, 1969). Protein concentrations were measured by the method of Bradford (1976), with bovine serum albumin as standard.

Administration of phenobarbital to chick embryos and hatched chicks

Phenobarbital (10 mg in 0.3 ml of 0.9% NaCl) was injected into the air space of eggs containing 17-day embryos, through a small hole which was then sealed with candle wax (Wishart & Dutton,

1975). Control eggs received solvent only. The eggs were incubated for a further 3 days before the livers were removed from the 19–20-day embryos. Hatched chicks were supplied with 0.25 g of phenobarbital/l in the drinking water for 3 days.

Purification of hepatic UDP-glucuronyltransferase

Liver (10 g) was obtained from 19-day embryos pre-treated with phenobarbital. Microsomal UDP-glucuronyltransferase was solubilized and partially purified by (NH₄)₂SO₄ fractionation as previously described (Burchell, 1977).

The 25–60%-satd.-(NH₄)₂SO₄ precipitate was dissolved in 0.05% (w/v) Lubrol/20 mM-Tris/HCl/5 mM-mercaptoethanol/20% (w/v) glycerol, pH 8.0 (buffer A), to give a yellow/red solution (10 ml). This extract was dialysed against 2 litres of buffer A for 16 h at 4°C. The dialysed fraction was applied to a DEAE-cellulose column (15 cm × 2 cm). UDP-glucuronyltransferase activity towards 1-naphthol was eluted at 20 ml/h with 100 ml of buffer A followed by a 200 ml linear gradient of 0–0.3 M-KCl in buffer A (see the Results section). Selected DEAE-cellulose fractions were concentrated approx. 15-fold by vacuum dialysis and the concentrates were dialysed against 200 vol. of buffer A adjusted to pH 7.4 (buffer B). UDP-glucuronyltransferase was further purified by affinity chromatography as previously described (Burchell, 1978), but with buffer B in the chromatographic method. Each purification fraction was reconstituted with phosphatidylcholine liposomes (1 mg/mg of protein) as previously described (Burchell, 1980) before assay of enzyme activities.

Slab-gel SDS/polyacrylamide-gel electrophoresis of various enzyme preparations was performed by the method of Laemmli (1970) on 7–9%-acrylamide gradient gels (15 cm × 15 cm) run at a constant 20 mA.

Chick-embryo hepatocyte monolayer cultures

Hepatocyte monolayer cultures were set up as described by Skea & Nemeth (1969) and Burchell *et al.* (1972). The isolated hepatocytes were resuspended (10⁶ cells/ml) in Eagle's Minimum Essential Medium containing 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], 2 mM-L-glutamine, 50 units of penicillin/ml, 50 µg of streptomycin/ml, 10% (w/v) foetal-calf serum (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) and 2 mM-sodium phenobarbital. The cell suspension was added into 5 cm culture dishes (Sterilin, Teddington, Middx., U.K.), 4 ml per dish, and cultures were incubated at 37°C in a humidified incubator in a CO₂/air (1:19) atmosphere. For preparation of microsomes (microsomal fractions), cells were washed with 0.25 M-sucrose. Then the washed cells (approx. 20%, w/v) resuspended in 0.25 M-sucrose

were homogenized in a Teflon/glass homogenizer and sonicated for 15 s (approx. 120 W) with a Dawe Soniprobe (Branson Instruments). Microsomal fractions were obtained by the differential-centrifugation procedure described by Burchell (1977).

Results

Assay of UDP-glucuronyltransferase activity towards various substrates

The 'latent' activity of hepatic microsomal UDP-glucuronyltransferase is well recognized, and the extent of this 'latency' may vary after drug or hormonal treatment of animals (Dutton & Burchell, 1977). Therefore, measurement of changes of enzyme activities resulting from drug pretreatment must be performed when the enzyme has been optimally activated, preferably by a non-ionic detergent (Dutton, 1980).

Optimal activation of hepatic microsomal UDP-glucuronyltransferase towards 1-naphthol was assessed by incubation of samples of the microsomes with various concentrations of detergent in the enzyme assay mixtures. Approx. 50 µg of microsomal protein was exposed to concentrations of Lubrol of 0.1–1.0% (w/v) in a final assay incubation volume of 200 µl. Optimal activation (approx. 12-fold) of UDP-glucuronyltransferase in 7-day hatched chick or 19–20-day-embryo liver microsomes was achieved with the ratio 1 mg of Lubrol/mg of microsomal protein. However, optimal activation (approx. 15-fold) of microsomal UDP-glucuronyltransferase from chick-embryo hepatocyte monolayer cultures was achieved by the presence of 0.5 mg of Lubrol/mg of microsomal protein.

Heterogeneity of hepatic UDP-glucuronyltransferase in hatched chick liver

Detergent concentrations which optimally activated microsomal UDP-glucuronyltransferase ac-

tivity towards 1-naphthol also proved to be optimal concentrations for measurement of glucuronyltransferase activities with other substrates. Table 1 shows that liver microsomes from untreated and phenobarbital-treated 7-day-old chicks exhibited high UDP-glucuronyltransferase activity towards three simple phenols, whereas testosterone was an extremely poor substrate and no activity was detectable with bilirubin as substrate. Thus a limited spectrum of activities could be detected in young chicks, which by comparison with the mammalian system may suggest limited heterogeneity of the enzyme at this stage of development (see the Discussion section).

Effect of phenobarbital or corticosterone acetate administration on UDP-glucuronyltransferase activities in chick-embryo liver

Hepatic microsomal UDP-glucuronyltransferase activity of untreated 19–20-day chick-embryo livers was extremely low or not detectable with five different substrates (Table 1). Treatment of embryonated eggs with phenobarbital caused up to a 50-fold increase in hepatic microsomal UDP-glucuronyltransferase activity towards 1-naphthol, 4-nitrophenol and 2-aminophenol (as previously reported; see Dutton, 1980), whereas activity towards bilirubin remained unchanged and that towards testosterone only slightly increased (Table 1). Thus phenobarbital stimulates the precocious appearance of higher (up to 3 times adult values) UDP-glucuronyltransferase activities than those detectable in the hatched chick liver.

Infusion of corticosterone acetate into embryonated eggs also increased UDP-glucuronyltransferase activity towards 1-naphthol some 20-fold (results not shown).

Expression of UDP-glucuronyltransferase activities in chick-embryo hepatocyte monolayer cultures

The isolation and culture of chick-embryo hepatocytes as cell monolayers (see the Experimental

Table 1. *Substrate specificity of chick liver microsomal UDP-glucuronyltransferase*

Microsomal pellets were resuspended in 0.25 M-sucrose, and samples (50 µg of protein) were treated with the optimal amount of Lubrol required to activate UDP-glucuronyltransferase maximally (see the Results section). The results shown are averages of at least three separate experiments, except for the data from phenobarbital-pretreated chick livers (each experiment involves the isolation of microsomes from at least six livers). Values in parentheses indicate the range of data obtained. Abbreviations: N.D., not detectable; PB, phenobarbital pretreated.

Substrate	UDP-glucuronyltransferase activity (nmol/min per mg of protein)						
	7-day hatched chick		19-day chick embryo		Embryo cell cultures		
	Control	+ PB	Control	+ PB	Control	+ PB	
1-Naphthol	18.7 (12.6–28.0)	78.6	1.23 (0.17–2.0)	62.7 (55.9–78.0)	37.9 (33.4–39.1)	40.7 (30.8–42.0)	
4-Nitrophenol	4.8 (1.8–7.9)	19.6	0.65 (0.0–1.1)	10.0 (8.4–11.6)	7.6 (3.1–10.0)	9.7 (6.0–11.1)	
2-Aminophenol	2.9 (0.8–4.9)	17.0	0.34 (3.1–0.58)	10.2 (5.8–13.9)	12.7 (4.7–21.1)	15.3 (10.2–17.5)	
Testosterone	0.05 (0.04–0.07)	0.11	0.03 (0.01–0.05)	0.14 (0.05–0.18)	0.11 (0.05–0.20)	0.13 (0.11–0.15)	
Bilirubin	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	

Table 2. *Purification of UDP-glucuronyltransferase from phenobarbital-treated chick embryo livers*
Data presented are from a single purification experiment. Numbers in parentheses are ranges of values obtained from at least five preparations. Enzyme activity was determined with 1-naphthol as acceptor substrate.

Fraction	Volume (ml)	Total protein (mg)	Specific activity (nmol/min per mg of protein)	Purification (fold)	Yield (%)
Lubrol supernatant	19	190	40	1	100
25–60% Satd.-(NH ₄) ₂ SO ₄	9	81	56 (53–90)	1.4	59
DEAE-cellulose eluate	3	13.5	119 (53–119)	2.9	21
UDP-glucuronic acid eluate from UDP-hexanolamine–Sephacel	4.8	0.21	2503 (1830–3717)	62	6.7

Fig. 1. *Isolation of chick-embryo liver UDP-glucuronyltransferase activity by DEAE-cellulose chromatography*
An (NH₄)₂SO₄ fraction was prepared, dialysed and applied to a DEAE-cellulose column (see the text). Fractions (1.5 ml) were collected at a flow rate of 20 ml/h and assayed for transferase activity towards 1-naphthol (■) after reconstitution with phosphatidylcholine liposomes (1 mg of phospholipid/mg of protein). ●, A₂₈₀ (protein); ····, 0–0.3 M-KCl gradient in buffer A. The hatched area represents the enzyme activity included in fractions 51–66, which were pooled, concentrated and used for further purification of the enzyme.

section) results in the spontaneous appearance of high UDP-glucuronyltransferase activity towards 2-aminophenol within 3 days, as previously reported (Skea & Nemeth, 1969). Addition of phenobarbital to the culture medium only caused a slight increase over the spontaneously developed enzyme activity (Burchell *et al.*, 1972). Here, we have determined that this UDP-glucuronyltransferase also exhibited activity towards 1-naphthol and 4-nitrophenol, but not towards bilirubin or testosterone (Table 1).

These results indicate that the expression of a similar UDP-glucuronyltransferase activity could be observed in monolayer cultures, hatched chicks and phenobarbital-treated chick embryos.

Purification of hepatic UDP-glucuronyltransferase from phenobarbital-treated chick embryo

Solubilized chick liver UDP-glucuronyltransferase activity could only be effectively measured

after reconstitution of the protein with phospholipid, even in the presence of the detergent Lubrol. Thus all purification fractions were reconstituted with phospholipids (see the Experimental section) before assay. Purification of UDP-glucuronyltransferase from 10 g of chick-embryo liver was then performed by the procedure described in the Experimental section. The results of this purification are shown in Table 2. The critical step in the purification was the selection of fractions from DEAE-cellulose (Fig. 1) for further purification by affinity chromatography on UDP-hexanolamine–Sephacel (see Burchell, 1978). Selection of pooled fractions 51–66 from DEAE-cellulose (Fig. 1) facilitated the purification of UDP-glucuronyltransferase to near homogeneity (see Fig. 2) in approx. 7% yield. Thus approx. 0.2 mg of purified enzyme was obtained for further studies. The proportions of activities of the purified enzyme

Fig. 2. *SDS/polyacrylamide-gel electrophoresis of chick-embryo liver microsomes and purified UDP-glucuronyltransferase*

Slab-gel electrophoresis was performed in a 7–9% linear gradient of separating gel (15 cm × 15 cm) and a 3% gel stack (2 cm × 15 cm) in the presence of 0.1% SDS (see the Experimental section). (a), Tracks 1 and 5, standards of known molecular weight (2 µg of each protein); 2, microsomes from untreated chick-embryo liver (approx. 100 µg of protein); 3, microsomes from phenobarbital-treated chick-embryo liver (approx. 100 µg of protein); 4, purified UDP-glucuronyltransferase (GT; 1 µg of protein). (b), Tracks 1 and 7, purified GT (1 µg of protein); 2 and 8, standards of known molecular weight; 3 and 4, microsomal fraction from chick-embryo liver cultures (approx. 100 µg of protein); 5 and 6, microsomal fraction from chick-embryo liver cultures exposed to phenobarbital (approx. 100 µg of protein). (c), Track 1, protein standards [albumin (mol.wt. 68 000), pyruvate kinase (57 000), fumarase (49 000) and aldolase (40 000)]; 2, equal-volume mixture of microsomes from cultured chick-embryo liver cells (see track 3, approx. 10 µg of protein) and purified GT (see track 4; 1 µg of protein). Gels were stained with Coomassie Brilliant Blue for 60 min and destained with acetic acid/methanol/water (7:5:43, by vol.). Direction of migration is from the top to the bottom. '68 K' etc. represent mol.wts. of 68 000 etc.

towards 1-naphthol, 4-nitrophenol and 2-aminophenol are similar to those exhibited by the starting microsomal preparation, i.e. approx. 7:1:1.

Identification and selective induction of the UDP-glucuronyltransferase protein in phenobarbital-treated chick-embryo liver cells

Microsomes from phenobarbital-treated chick embryos and chick-embryo hepatocyte monolayer cultures exposed to phenobarbital were analysed by SDS/polyacrylamide-gradient-gel electrophoresis (see the Experimental section). Comparison of the hepatic microsomes with purified UDP-glucuronyltransferase allowed the identification of the enzyme as a 53 000-Da protein in the microsomal preparation (Fig. 2). Enrichment of these microsomal preparations by mixing with the purified enzyme confirmed the location and that the accumulation of this protein occurred after exposure of hepatocytes to phenobarbital (Fig. 2).

Discussion

Mammalian hepatic UDP-glucuronyltransferase activities have been shown to be due to the existence of a family of differentially inducible enzymes (Burchell, 1981). In contrast, the substrate specificity of neonatal-chick liver or phenobarbital-treated chick-embryo liver reported here was remarkably limited. Activity towards bilirubin was not detectable, presumably because this particular form of UDP-glucuronyltransferase is not required and may not be present in avian liver, where water-soluble biliverdin is excreted as the terminal haem breakdown product (Minkowski & Naunyn, 1886; Lemberg & Legge, 1949).

Only very low UDP-glucuronyltransferase activity towards testosterone was detected, and this was only slightly increased by exposure of liver cells to phenobarbital, as previously reported (Burchell, 1974). In fact, steroids appear to be conjugated with sulphate and not with glucuronide in chick liver

(Layne, 1970), which accounts for the low steroid UDP-glucuronyltransferase activity. Thus the functional heterogeneity of neonatal-chick liver UDP-glucuronyltransferase as so far determined is confined to the formation of phenol glucuronides; 1-naphthol, 2-aminophenol, 4-nitrophenol were glucuronidated by a single form of UDP-glucuronyltransferase, which, as reported here, can be purified to near homogeneity from chick-embryo liver.

Drug- or hormone-mediated induction of an enzyme has been previously defined as 'an increase in enzyme activity which requires continuous protein synthesis' (Dutton & Burchell, 1974). The protein synthesized may be the enzyme itself, or another protein which affects the rate of turnover of the enzyme (Dutton & Burchell, 1974). Activation of a membrane-bound enzyme by modification of its membrane environment may be incorrectly termed as enzyme induction (Dutton & Burchell, 1977). Thus it is important that membrane-bound enzyme must be optimally activated before measurement of enzyme activity, which may indirectly indicate that enzyme induction has occurred (Dutton, 1975). However, induction of an enzyme should be measured by a change in the concentration of the enzyme protein.

We have previously observed that the precocious development of UDP-glucuronyltransferase in chick-embryo liver cultures was dependent on protein synthesis (Burchell *et al.*, 1972). Subsequent advances in the isolation and characterization of mammalian liver UDP-glucuronyltransferase(s) in this and other laboratories (see Burchell, 1981) have provided methodology which can be used to purify the chick-embryo liver enzyme. Thus we have purified a UDP-glucuronyltransferase from as little as 10g of chick-embryo liver and we have used this purified enzyme as a 'standard' protein to show that phenobarbital treatment of liver cells results in the accumulation of large quantities of UDP-glucuronyltransferase protein. Presumably the precocious appearance of this enzyme was due to synthesis *de novo*, although phenobarbital may also decrease its rate of degradation (Burchell *et al.*, 1972).

The effect of drugs on the turnover of microsomal proteins in chick-embryo hepatocytes has been studied (Althaus & Meyer, 1981), although the function of the proteins examined was not known. A number of microsomal proteins were assumed to be multiple forms of cytochrome P-450 (Althaus *et al.*, 1979; Althaus & Meyer, 1981). Indeed, routine screening of microsomal proteins by SDS/polyacrylamide-gel electrophoresis for the drug-mediated appearance of a particular cytochrome P-450 is obviously confused by the possible induction of other microsomal proteins such as UDP-glucuronyltransferase. This is particularly true in chick-embryo liver microsomes, where pheno-

barbital induces the accumulation of large amounts of UDP-glucuronyltransferase, a protein of mol.wt. 53000. Althaus & Meyer (1981) have determined that the rate of degradation of a 54000-Da microsomal protein was not altered by treatment of chick-embryo hepatocytes with β -naphthoflavone; the half-life of this inducible protein was estimated to be approx. 10h. Previous estimates of the half-life of chick-embryo liver UDP-glucuronyltransferase were much longer, approx. 65h (Burchell *et al.*, 1972). Here we have shown that UDP-glucuronyltransferase is a phenobarbital-inducible protein of mol.wt. 53000. [35 S]Methionine can be incorporated into this protein during liver cell culture (B. Burchell, unpublished work). Thus the turnover of this enzyme after drug or hormone treatment of liver cells can now be reassessed. Indeed, cultured chick-embryo liver cells should prove to be an excellent system for the study of the regulation of UDP-glucuronyltransferase.

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