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RELEASE BY PHENOBARBITAL OF THE REPRESSION OF UDP-GLUCURONYLTRANSFERASE ACTIVITY *IN OVO*

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Abstract—UDP-Glucuronyltransferase activity in chick-embryo liver and kidney, known to be induced by hatching or by culture, appears to be repressed *in ovo*. Injection of phenobarbital into the egg overcomes this repression. Chick-embryo liver and kidney cells can thus exhibit high transferase levels whilst still *in ovo*. Response to phenobarbital by liver tissue occurs *in situ* or when grafted on to the chorioallantoic membrane, and can be elicited by 96 hr of age. The ability of phenobarbital to over-ride the natural regulation of liver UDP-glucuronyltransferase development *in ovo* is contrasted with its inability *in utero*. Overall glucuronidation, as well as the transferase, is elicited by phenobarbital *in ovo*; differences between regulation of glucuronidating and hydroxylating enzymes are noted.

UDP-Glucuronyltransferase [E.C. 2.4.1.17] activity is first detectable in chick-embryo liver at 7 days' incubation [1]. Activity reaches a low peak around 14 days and falls virtually to zero before rising rapidly on hatching at 21 days. Adult levels are reached within 24–48 hr of hatching and are some 10 times the maximum levels achieved *in ovo*. If embryo liver cells or fragments are cultured in a chemically-defined medium, UDP-glucuronyltransferase activity rises precociously from near-zero to values 5–10 times those found in "adult" chick liver [2, 3]. This striking rise requires protein synthesis [4]. The culture medium does not induce the enzyme [5]. Moreover, culture *in ovo* on the chorioallantoic membrane prevents the rise [3]. Therefore simple removal from the egg initiates the rise. An embryonic environment appears to repress development of the enzyme towards all xenobiotic substrates examined.

Before investigating the endogenous factors responsible for low activity of UDP-glucuronyltransferase *in ovo* and for its natural development on hatching, it was necessary to know if premature appearance of the enzyme and its rapid increase to high values were indeed possible in the embryonic environment. Exposure to phenobarbital accelerates induction of UDP-glucuronyltransferase by culture [4] and by hatching [6]. We report here the effect of phenobartital on the activity of the transferase and associated enzymes *in ovo*. A preliminary note on part of this work has been published [5].

MATERIALS AND METHODS

Embryonated White Leghorn eggs were obtained at day 0 and incubated at 39° and 55 per cent relative humidity. Compounds were injected into the air space through a small hole which was then sealed with paraffin wax. Control eggs received solvent only. Culture methods were as described previously [4]. Chorioallantoic grafting was as described by New [7].

Chemicals were as described previously [1, 4].

Assays for UDP-glucuronyltransferase were with *o*-aminophenol [3], bilirubin [8], 4-methylumbelliferone [9], *p*-nitrophenol [10], *p*-nitrothiophenol [11], oestriol [12]; glucuronidation of *o*-aminophenol by slices was as described previously [13]. Other enzymes assayed were aniline hydroxylase [14] (E.C. 1.14.1.1), benzpyrene hydroxylase [15] (E.C. 1.14.1.1), NADPH-cytochrome *c* reductase [16] (E.C. 1.6.2.3), cytochrome P-450 [17], glucose-6-phosphatase [10] (E.C. 3.1.3.9), phenylalanine hydroxylase [18] (E.C. 1.14.3.1). Protein was estimated as previously [4], and phenobarbital by g.l.c. [19].

RESULTS

Effect of injected phenobarbital. In an attempt to overcome the *in ovo* repression of UDP-glucuronyltransferase activity, phenobarbital was injected in B.S.S. (Basal Salt Solution [20]) into the air space. The drug increased hepatic UDP-glucuronyltransferase activity markedly within a few days. Figure 1 illustrates the response to injection of 20 mg phenobarbital on day 5. Enzyme activity increased with time of exposure to this single dose, and by day 18 reached 10 times adult values. The embryos did not hatch successfully, but livers examined at day 21 still contained high enzyme levels.

If 25 mg phenobarbital were injected on day 5, assay by g.l.c. 3 days later showed 16 μg of the drug in the embryo and 4·4 mg in the extra-embryonic fluids.

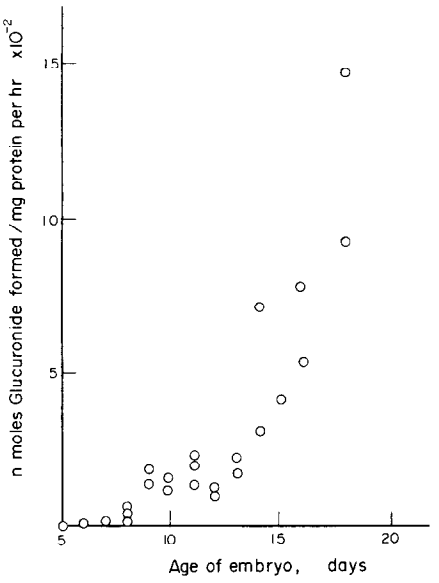

Fig. 1. Increase of embryo-liver UDP-glucuronyltransferase activity following a single injection of phenobarbital. Phenobarbital (20 mg) was injected into eggs on day 5, and transferase activity towards *o*-aminophenol assayed in the embryo liver on subsequent days indicated. Points represent a single experiment and are means of duplicate assays differing by less than 10 per cent. Methods as in text.

Total phenobarbital content of the embryo increased with age, but concentration of the drug (mg/wet wt tissue) remained approximately constant.

The response was dose-dependent (Fig. 2). The magnitude of enzyme response increased with age (Table 1). Older embryos (>17 days) exposed to large doses (>10 mg injected) died at hatching. Younger embryos

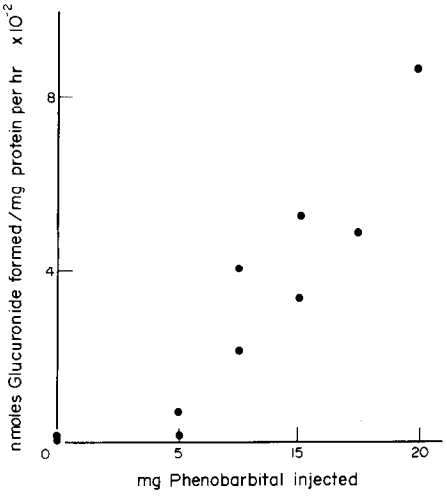

Fig. 2. Dependence of embryo-liver UDP-glucuronyltransferase activity on dose of phenobarbital injected. Phenobarbital (0–20 mg) was injected on day 17, and transferase activity assayed on day 20. Results derived as for Fig. 1.

Table 1. Effect on embryo-liver UDP-glucuronyltransferase of exposure to phenobarbital at various ages

Age of embryo on injection (days)	Glucuronide formed (nmoles/mg protein/hr) (a)	(b)
5	2·4	2·4
11	48·6	61·2
17	291·6	454·2

Ten mg phenobarbital was injected into the air-space on days shown, and 3 days later enzyme activity in the embryo liver was assayed towards *o*-aminophenol. Results are shown for 2 eggs (a) and (b) at each age, and are means of duplicate assays differing by less than 10 per cent. Methods as in text.

with smaller doses hatched successfully; their transferase levels, if already increased to adult values or above, did not greatly increase further on hatching.

Response to injected drug was detectable very early in life. If eggs were injected with 50 mg phenobarbital after 1 day of incubation, the visceral area (containing developing liver, oesophagus, pancreas and stomach) displayed transferase activity 72 hr later; for example 24·0 nmoles *o*-aminophenol were conjugated/mg "visceral" protein/hr compared with zero in control embryos.

Phenobarbital exerted its effect when injected into air space, yolk sac or embryo itself; the latter route gave a 2-fold greater response, but was frequently lethal. If the drug was suspended in arachis oil before injection into the air space, little increase in UDP-glucuronyltransferase resulted over 3 days.

At standard concentrations of 6 mM UDP-glucuronic acid and 0·6 mM *o*-aminophenol, enzyme activity was linear for the incubation period. When assays were conducted with 1, 3 and 10 mM UDP-glucuronic acid and 0·1, 0·3 and 1 mM *o*-aminophenol, liver UDP-glucuronyltransferase activity from treated eggs was always greater than that from controls by a similar degree.

Phenobarbital itself did not activate UDP-glucuronyltransferase *in vitro*; it inhibited at higher concentrations (50 mM). Mixed-homogenate experiments with livers from treated and control eggs gave no evidence of activators formed during metabolism of phenobarbital. Although pulsing with inhibitors of protein synthesis is not practicable *in ovo*, these results, together with the gradual increase of activity, suggest that induction of the transferase is a more likely explanation than activation.

As well as the transferase itself, overall glucuronidation also was increased in embryo liver by phenobarbital. For example, 25 mg drug were injected on day 5, and liver slices taken on day 17; from 2 injected eggs, slices conjugated 13·9 and 9·6 nmoles *o*-aminophenol/mg protein/hr respectively. No conjugation occurred in comparable slices from control eggs.

Table 2. Effect of phenobarbital and of culture on embryo-kidney UDP-glucuronyltransferase

Expt. no.	Age of fresh tissue (days)	*o*-Aminophenyl glucuronide formed (nmoles/mg protein/hr) (a)	(b)	(c)	(d)	(e)
1	6	0	74·4	117·8	—	—
2	12	3·2	31·0	—	—	—
3	16	4·8	14·0	32·0	—	—
4	4	—	—	—	0	7·7 (30)
5	11	2·0	—	—	0·7	18·0 (10)

UDP-Glucuronyltransferase of embryo kidney was assayed (a) in fresh tissue; (b) in tissue from (a) cultured for 4 days; (c) as for (b) but cultured with 5·5 mM phenobarbital in medium; (d) in tissue from eggs injected with B.S.S. at age of (a) and then incubated for 3 days after injection; (e) as for (d), but injected with phenobarbital (mg shown in parentheses). Methods as described in text. Mesonephros was employed in Expts. 1 and 4, metanephros in Expts. 3 and 5, and mixed (mostly metanephros) in Expt. 2. In each experiment, pooled tissue from several eggs was used.

Effect of phenobarbital at other sites in ovo. Injection of phenobarbital into eggs increased UDP-glucuronyltransferase in embryonic kidney, both mesonephros and metanephros (Table 2). Simple culture of these organs also increased the enzyme, phenobarbital in the medium enhancing the increase.

In duodenum and skin from injected eggs, increase of the enzyme was minimal; in spleen, brain and lungs enzyme did not appear. Transferase was also absent from allantoic and chorioallantoic membranes, yolk sac and yolk whether the drug had been injected or not. Membrane strips, incubated as tissue slices, gave no evidence of glucuronidation.

As phenobarbital clearly overcame *in ovo* repression of the liver enzyme in the intact embryo, its effect was studied on embryo liver cultured on the chorioallantoic membrane; in uninjected eggs the enzyme remains uninduced by such culture [3]. Fragments of 6-day embryo liver were cultured for 3 days on the membrane of 11-day eggs which received injections of (a) phenobarbital or (b) B.S.S. From (a) eggs, cultured fragments conjugated (i) 11·8 and (ii) 6·5 nmoles *o*-aminophenol/mg protein/hr, compared with (i) 0·6 and (ii) 1·2 from (b) eggs. Phenobarbital thus overcomes repression of embryonic liver UDP-glucuronyltransferase *in ovo* whether the liver remains *in situ* or is removed from the embryo.

Effect of phenobarbital with other substrates. Injection of eggs with phenobarbital increased UDP-glucuronyltransferase activity in a dose-dependent manner for the following other substrates (expressed as nmoles glucuronide/mg protein/hr, controls in parentheses); *p*-nitrophenol 36 (0); *p*-nitrothiophenol 68 (0); oestriol 8·0 (1·4). No embryo-liver transferase activity, with or without phenobarbital injection, could be demonstrated for the following substrates of the mammalian-liver enzyme: bilirubin, chloramphenicol (B. Burchell, personal communication), serotonin (J. Leakey, personal communication), 4-methylumbelliferone. Culture of embryo liver did not induce activity towards these compounds and activity was also absent from liver of hatched chickens.

Effect of phenobarbital on other enzymes. Certain associated microsomal hydroxylating enzymes increased with the transferase *in ovo* after injection of phenobarbital into 16-day eggs. Within 3 days, UDP-glucuronyltransferase had increased in microsomes × 20, cytochrome P-450 × 9, aniline hydroxylase × 3, and benzpyrene hydroxylase × 1·7; NADPH-cytochrome *c* reductase and glucose-6-phosphatase remained unchanged at approximately adult levels. 3-Amino-1,2,4-aminotriazole (up to 20 mg in B.S.S.) injected along with phenobarbital reduced or abolished the response of aniline hydroxylase to phenobarbital but increased that of the transferase. Phenylalanine hydroxylase which unlike UDP-glucuronyltransferase responds to hydrocortisone *in ovo* (G. Wishart and M. Sudjić, unpublished work) did not respond to phenobarbital, even when already induced by hydrocortisone.

DISCUSSION

UDP-Glucuronyltransferase is induced by hatching or by culture. Its induction on culture is prevented if liver fragments are cultured *in ovo* on the chorioallantoic membrane, but occurs if these cells are subsequently cultured in a dish; if fragments are cultured in a dish and then placed on the membrane their further development of transferase ceases [3]. The embryonic environment presumably represses development of UDP-glucuronyltransferase. The enzyme is induced also by exposure of liver to phenobarbital, both in culture [4] and in the hatched bird [6]. As the culture-induced, phenobarbital-induced and naturally-developing enzymes possess similar kinetic properties after various treatments [6], results obtained with phenobarbital-induced transferase may be considered relevant to the naturally-developing enzyme.

The findings reported above indicate that embryo liver cells can exhibit UDP-glucuronyltransferase activity at high levels *in ovo*, that they can respond *in ovo* even when grafted extra-embryonically, and that the response can be elicited in the hepatic area as early as 96 hr of age. Embryonic kidney tissue also displays precocious transferase activity on culture or when

exposed to phenobarbital within the egg. Phenobarbital can therefore override the natural regulation of transferase development for these tissues *in ovo*, and at a very early age. This contrasts notably with the situation *in utero*; even when mammalian foetal liver possesses the same high phenobarbital levels as maternal liver, transferase development in foetal liver is not increased by the drug until just before birth [21] (B. Burchell and G. J. Dutton, unpublished work). As phenobarbital has a similar lack of effect on foetal transferase when foetal liver is cultured in a dish [21] or *in ovo* on the chorioallantoic membrane (G. J. Wishart, unpublished work) a maternal signal may be required before a foetal response to the drug can be evoked; or the inducer may be a metabolite of phenobarbital which can be made by the embryonic, but not by the foetal, liver. Whatever the mechanism, chick embryo liver and kidney is competent to respond by at least the first 3·5 days of development.

It is not surprising that overall glucuronidation increases *in ovo* following phenobarbital injection, for unlike the mammalian foetus chick embryo contains adult levels of liver UDP-glucuronic acid [1] and UDPG dehydrogenase [22], and phenobarbital injection of eggs increases the latter enzyme 5-fold [22].

Induction of hydroxylating and glucuronidating systems can be differentiated by using *in ovo* and culture techniques [23] and the findings with aminotriazole reported above further suggest a difference in developmental control between the two systems. The *in ovo* technique is thus usefully intermediate between the intact animal and cultured tissue.

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