Precocious Development of Uridine Diphosphate Glucuronosyltransferase Activity during Organ Culture of Foetal Rat Liver in the Presence of Glucocorticoids

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1. Precocious development of mammalian UDP-glucuronosyltransferase (EC 2.4.1.17) induced by endogenous compounds of known chemical composition is reported for the first time. 2. This development occurs in cultured explants of foetal rat liver when exposed to corticosteroids possessing a pregn-4'-ene structure and a hydroxy or an oxo group at C-11. 3. Explants from 14-day fetuses cultured for 3 days in a chemically defined medium containing dexamethasone exhibited transferase activities towards *o*-aminophenol within adult male values. Those liver transferase activities attained *in utero* by 17 days were still negligible. 4. Evidence from several approaches indicated that the explants required glucocorticoids for expression of the transferase, not for maintenance of viability. 5. Glucocorticoid-dependent stimulation of transferase activity required incorporation of L-[¹⁴C]leucine into protein, as judged from the pulsing of cultures with cycloheximide. 6. The relevance of these culture experiments to the situation *in vivo* is discussed.

Glucuronidation is the principal pathway of detoxication in mammals. It is virtually undetectable in the early foetus, but develops perinatally. This development can be accelerated somewhat by the administration of drugs, but the natural mechanisms controlling its onset have remained unknown (for references, see Dutton & Burchell, 1977).

We have demonstrated that, in chick-embryo liver, glucuronidation and UDP-glucuronosyltransferase (EC 2.4.1.17), the rate-limiting enzyme of glucuronidation in that tissue, can be precociously stimulated from negligible to adult values by treatment *in ovo* with corticotropin or certain glucocorticoids (Leakey *et al.*, 1976). We have proposed that this mechanism is responsible for the natural surge of the transferase activity on hatching (Wishart & Dutton, 1975; Leakey *et al.*, 1976).

We have extended these studies to foetal rat liver. We report here the precocious development of the transferase up to or above maternal values, in cultured explants of foetal rat liver. Glucocorticoids are required for this process, and protein synthesis appears to be involved. Preliminary accounts of part of this work have already been published (Wishart & Dutton, 1976; Wishart *et al.*, 1976).

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Materials and Methods

Cycloheximide and all steroids, natural and synthetic, were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. L-[1-¹⁴C]-Leucine (60mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Rats were Wistar strain, time-mated. Organ culture, in a protein-free defined medium, was as described for chick-embryo liver (Burchell *et al.*, 1972). UDP-glucuronosyltransferase activity towards *o*-aminophenol was assayed by the method previously used (Leakey *et al.*, 1976), but because the transferase in foetal liver, unlike that from chick embryos, exhibits latency and age-dependent activation (Leakey & Donald, 1976; Dutton *et al.*, 1976) the following modifications were used: the enzyme source was a homogenate (5%, w/v), prepared by six strokes of a manually operated glass-Teflon homogenizer; in each experiment, transferase was assayed in a series of digitonin concentrations [0, 0.04, 0.08, 0.12, 0.16 and 0.20% (w/v) respectively, dissolved in water at room temperature (18°C)]. Assay duplicates, when performed, did not differ by more than $\pm 5\%$; usually single samples from duplicate cultures were used, each assayed with and without added UDP-glucuronic acid. Reaction rates were linear with respect to time and enzyme concentration. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Results are expressed as means $\pm$ s.e.m. unless otherwise indicated.

Incorporation of L-[1- ^{14} C]leucine into protein was measured as described by Burchell *et al.* (1972), except that precipitated protein was collected by centrifugation; 3.5 ml of aqueous suspension of this protein or 3.5 ml of trichloroacetic acid-soluble fraction was added to 11.5 ml of Aquasol (New England Nuclear Corp., Boston, MA, U.S.A.) before counting the radioactivity for 10 min in a Nuclear-Chicago Unilux II liquid-scintillation counter, giving values of 1500–6500 d.p.m. against a background of 30–50 d.p.m. Counting efficiency was 50–60%, measured from channel ratios. All statistical representations are expressed as S.E.M. to 95% confidence limits.

Results

Natural development of UDP-glucuronosyltransferase activity towards o-aminophenol

When homogenates were treated with digitonin to optimal activation [0.08–0.16% (w/v) digitonin, final concn.], transferase activity was found to develop progressively from negligible values on day 17 of gestation to a peak just before birth on day 21. Peak values were some five times those in optimally activated preparations from maternal liver. In homogenates untreated with digitonin, or activated suboptimally with the detergent, a similar pattern was observed, but the values were often too low for accurate assay. This similarity persisted through all subsequent experiments, and although the full range of digitonin concentrations was always used as a precaution, results quoted below are those at the optimal concentration, which remained 0.08–0.16% (w/v).

Development of UDP-glucuronosyltransferase activity in cultured foetal liver

Foetal-liver explants were cultured as described in the Materials and Methods section, with and without 2 μ M-dexamethasone in the culture medium. In explants from 15-day foetuses, cultured with dexamethasone for 4 days, the surge in transferase activity appeared some 24 h before it would have been evident *in utero*, and continued to develop at much the same rate as *in utero* (Fig. 1a). Without added dexamethasone no increase in transferase activity was seen throughout the 4 days of culture. If explants from 14-day foetuses were similarly cultured, transferase development in the presence of dexamethasone was even more obviously stimulated precociously, reaching, after 3 days culture, values at least seven times those attained *in utero* at 17 days; these values lay within the adult male range of 30–40 nmol of *o*-aminophenol glucuronidated/h per mg of protein, and above maternal values of 10–20

Fig. 1. Effect of dexamethasone on development of UDP-glucuronosyltransferase activity

UDP-glucuronosyltransferase activity towards *o*-aminophenol, assayed as described in the Materials and Methods section, was measured in foetal rat liver *in utero* (Δ) and as explants cultured with ($\bullet$) or without ($\circ$) dexamethasone in the medium as described in the text. (a) illustrates 15-day liver, and (b) 14-day liver, cultured for 1–4 further days, compared with liver remaining *in utero* throughout that period. Age of the foetal liver, whether *in utero* or in culture, is expressed as days. S.E.M. values are shown, with numbers of experiments (each from a separate culture of pooled foetal liver) in parentheses.

of these units. In control cultures not exposed to dexamethasone the activity again remained low. Cortisol reproduced the effect of dexamethasone, e.g. 14-day foetal livers exposed to 2 μ M-cortisol reached by day 17 a transferase activity of 25.8 μ mol of *o*-aminophenol glucuronidated/h per mg of protein.

Evidence for specific stimulation of UDP-glucuronosyltransferase activity

It was necessary to know whether the glucocorticoids specifically increased transferase activity, or

whether they merely maintained viability of the cultured explant and so allowed it to manifest a culture-stimulated development of transferase. Evidence for the specific nature of the stimulation is as follows.

Delayed exposure to dexamethasone. Explants of 15-day foetal liver were cultured for 2 days in a control dexamethasone-free medium and then placed in one containing dexamethasone. Transferase activity, previously negligible, developed over the next 2 days at a similar or even greater rate in the transferred explants as in fresh 15-day explants cultured for 2 days in dexamethasone immediately on excision; in one example transferase activity increased in the last 2 days to 27.3 nmol of *o*-aminophenol glucuronidated/h per mg of protein.

Protein loss. This was examined to ascertain if the apparent increase in transferase activity could have been due to a gross loss of non-transferase protein and if the dexamethasone-treated cultures lost more, or less, total protein than the control cultures. Protein was assayed in a pooled sample of 15-day foetal livers on successive days of a 4-day culture period. Of the total protein remaining after day 1 of culture without dexamethasone, 91, 79 and 61% remained after days 2, 3 and 4 respectively; when cultured with dexamethasone the corresponding percentages were 87, 78 and 66. To obtain these values explants from between five and nine culture dishes (five explants per dish) were taken each day and pooled. Therefore the difference in specific transferase activity cannot be accounted for by loss of non-transferase protein.

Rate of incorporation of amino acids. Incorporation of L-[1-¹⁴C]leucine into explant protein (for details, see under 'Evidence for induction of UDP-glucuronosyltransferase') was similar whether dexamethasone was present or not. Cultures lacking dexamethasone (24) were compared with 30 cultures exposed to the glucocorticoid over 4 days of culture; a minimum of five culture dishes (each with five explants from the pooled livers of several litters) were examined each day for incorporation of label. With dexamethasone absent, incorporations of protein (expressed as $10^{-3} \times \text{d.p.m./h per mg of protein}$) for the 4 days successively were 2.94 ± 0.30 (5), 2.64 ± 0.23 (6), 2.57 ± 0.34 (6) and 2.24 ± 0.15 (7); with dexamethasone present, the corresponding values were similar, at 2.90 ± 0.20 (7), 3.08 ± 0.08 (9), 2.76 ± 0.25 (9) and 2.68 ± 0.14 (9) (numbers of experiments are given in parentheses). No time-dependent trend was evident in either case.

Histology. Explants of 15-day foetal liver cultured for 3 days in a dexamethasone-containing medium were indistinguishable from those cultured without added glucocorticoids, when examined by optical microscopy after staining with Haematoxylin and Eosin.

Fig. 2. Effect of pulsing with cycloheximide on development of UDP-glucuronosyltransferase activity and on incorporation of L-[1-¹⁴C]leucine into protein

Assay methods are as described in the Materials and Methods section; 15-day foetal-rat liver explants were cultured over several days with $2 \mu\text{M}$ -dexamethasone as described in the text. After 20h of culture the medium was pulsed with cycloheximide for 5h. Control cultures were pulsed similarly, but with culture medium instead of cycloheximide. Transferase activity (a) and uptake of label into protein (b) were assayed at the times shown: (○) test and (●) control cultures. The same pool of livers was used for amino acid uptake during cycloheximide pulsing as for most of the transferase assays under those conditions. S.E.M. values are shown, with numbers of experiments (each from a separate pool of litters) in parentheses. The period of pulse is indicated by the solid horizontal bar on the abscissae.

Evidence for induction of UDP-glucuronosyltransferase

Activations of the transferase by different procedures are not usually additive (see Dutton &

Table 1. *Effect of steroids on foetal-rat liver UDP-glucuronosyltransferase*

Steroids, or their acetates, were dissolved in ethanol and added to the culture medium, to give a final concentration of 1% (v/v) ethanol and 2 μ M-steroid. The following steroids or their acetates did not evoke transferase activity in the explant at this concentration or at 20 μ M; deoxycorticosterone (21-hydroxypregn-4-ene-3,20-dione); pregnenolone (3 β -hydroxypregn-5-en-20-one); progesterone (pregn-4-ene-3,20-dione); tetrahydrocortisol (3 α ,11 β ,17 α ,21-tetrahydroxy-5 β -pregnan-20-one); oestriol [oestra-1,3,5(10)-triene-13,16 α ,17 β -triol]; testosterone (17 β -hydroxyandrost-4-en-3-one). S.E.M. values are given, for four experiments.

Steroid	<i>o</i> -Aminophenol glucuronidated (nmol/h per mg of protein)
None	3.2 $\pm$ 0.5
Corticosterone (11 β ,21-dihydroxy-pregn-4-ene-3,20-dione)	21.7 $\pm$ 1.5
Cortisol (11 β ,17,21-trihydroxy-pregn-4-ene-3,20-dione)	31.0 $\pm$ 5.0
Cortisone (17 α ,21-dihydroxy-pregn-4-ene-3,11,20-trione)	28.3 $\pm$ 5.4
Dexamethasone (9-fluoro-11 β ,17,21-trihydroxy-16 α -methylpregna-1,4-diene-3,20-dione)	20.7 $\pm$ 3.5
Prednisolone (11 β ,17 α ,21-trihydroxypregna-1,4-diene-3,20-dione)	20.0 $\pm$ 3.0

Burchell, 1977). As the observed stimulation of transferase development was evident after optimal and after 'zero' activation by digitonin, it was not likely to be due to activation by glucocorticoids of a latent enzyme. The requirement of amino acid incorporation for the process was therefore examined.

When L-[1- ^{14} C]leucine was added to the medium in which 15-day foetal-liver explants were being cultured, radioactivity reached a concentration in the tissue leucine pool which remained constant for 4h; specific radioactivity of the protein increased linearly with respect to time for 3h. Specific radioactivities of tissue leucine pool and protein were measured 90min after addition of L-[1- ^{14} C]leucine to the medium on each day during the 4-day culture and were found not to change during culture. Similar explants were then cultured with dexamethasone and at 20h were pulsed for 5h with cycloheximide at a final concentration of 3.5 μ M. They were examined 1, 2, 3 or 4 days later for transferase activity (Fig. 2a) and L-[1- ^{14} C]leucine incorporation into protein (Fig. 2b). Incorporation of ^{14} C into protein was inhibited by two-thirds during the pulse, and the rate of increase of transferase activity had fallen to approximately the same extent when measured 24h later. At 48h after pulsing, both leucine incorporation and transferase development (i.e. rate of increase of transferase activity) had

recovered to their pre-pulse values. Dependence of the increase of transferase activity on leucine incorporation into protein is therefore likely, and is consistent with synthesis of the enzyme *de novo* in the presence of the glucocorticoid.

Specificity of steroid hormones stimulating precocious development of the transferase

Explants were cultured separately with several steroids (Table 1). Only those C-21 steroids possessing the pregn-4'-ene or pregna-1,4-diene structure with a hydroxyl or an oxo group at C-11 evoked precocious activity of UDP-glucuronosyltransferase towards *o*-aminophenol when present in the culture medium of liver explants from 15-day foetal rats.

Discussion

The above results indicate that in cultured liver from foetal rats, as in similar preparations from chick embryos (Leakey *et al.*, 1976), glucocorticoids can stimulate development of UDP-glucuronosyltransferase activity towards *o*-aminophenol. Once possible latency of the mammalian enzyme is allowed for, this phenomenon is more readily seen in the rat tissue, for on simple culture without glucocorticoids it does not exhibit the 'spontaneous' induction of the enzyme evident in cultured chick-embryo preparations (Ko *et al.*, 1967; Scherer, 1975). As with chick-embryo liver, the steroids effective with foetal rat liver all possess a pregn-4'-ene (or pregna-1,4-diene) structure and either an oxo or a hydroxy group at C-11. Their dependence on protein synthesis for their stimulatory effect, only inferred from results with chick embryo, is readily deduced from the work with foetal rats. The stimulation therefore appears to be due to induction, i.e. to accumulation of new molecules of the catalytic entity, rather than to simple activation of a pre-existing latent enzyme.

MacDonnell *et al.* (1975) query the validity, for extrapolation to conditions *in vivo*, of results with organ explants; they cite a gross loss of soluble protein and an only partial response to hormones, including glucocorticoids, among their evidence for the functional (though not necessarily histological) deterioration of certain cell populations during organ culture. MacDonnell *et al.* (1975) used liver from 20-day rat fetuses. Our own results suggest that younger liver is cultured more successfully. Moreover, the transferase is a particulate protein. Nevertheless, the criticism of MacDonnell *et al.* (1975) is valuable. It particularly limits the significance of a negative finding as a result of organ culture. A positive result such as ours, however, could also arise from conditions of culture and bear no direct relevance to the situation *in vivo*; for example, the 'spontaneous' induction of UDP-glucuronosyltransferase mentioned above (Ko *et al.*, 1967; Skea &

Nemeth, 1969; Scherer, 1975) or of 'adult' plasma proteins (Grieninger & Granick, 1975) in cultured explants or cells of chick-embryo liver. Also, glucocorticoids may somehow preserve in culture the inherent capacity of the cell to express the transferase, perhaps by stabilizing a certain structure of the endoplasmic reticulum necessary for development of the enzyme.

It is therefore significant that glucocorticoids will also stimulate the transferase in foetal rat liver *in utero*, when injected maternally (Wishart & Dutton, 1977a), and to much the same extent as observed in culture.

Our present results indicate that glucocorticoids act directly on the foetal rat liver to stimulate the transferase, and therefore that the findings *in utero* (Wishart & Dutton, 1977a) can result from direct action of glucocorticoids on the foetal liver *in utero* and not after their metabolism in the maternal or extrahepatic foetal compartment.

We have reported here studies with one species and one substrate. Preliminary work suggests (Wishart *et al.*, 1977; Wishart & Dutton, 1977b) that those activities of UDP-glucuronosyltransferase which reach a peak in the rat prenatally or at birth resemble glycogen synthetase (Jacquot *et al.*, 1973) in being precociously evoked by glucocorticoids. Transferase activities developing postnatally [the 'neonatal' cluster of Greengard (1971)] seem to require different or additional factors, both in culture and *in utero*.

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