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The role of glycogen synthase phosphatase in the glucocorticoid-induced deposition of glycogen in foetal rat liver

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1. The mechanism that underlies the induction of glycogen synthesis in the foetal rat liver by glucocorticoids was reinvestigated in conditions where the accumulation of glycogen is either precociously induced with dexamethasone or inhibited by steroid deprivation. It appears that glucocorticoids act as the physiological trigger for glycogen synthesis by inducing both glycogen synthase (a known effect) and its activating enzyme, glycogen synthase phosphatase. 2. The activity of glycogen synthase phosphatase in adult liver stems from the interaction of two protein components [Doperé, Vanstapel & Stalmans (1980) *Eur. J. Biochem.* **104**, 137–146]. Two independent experimental approaches indicate that the cytosolic 'S-component' is already well developed in the foetal liver before the onset of glycogen synthesis. The manifold glucocorticoid-dependent increase in synthase phosphatase activity during late gestation must be attributed to the specific development of the glycogen-bound 'G-component'.

Glycogen accumulates steadily in the liver of several foetal mammals during the last fifth of gestation (Shelley, 1961). The process has been investigated most extensively in the rat (for a review, see Stalmans & Laloux, 1979). It appears to be triggered physiologically by a transiently increased secretion of glucocorticoids by the foetal adrenals and involves protein synthesis.

The concentration of 'total' glycogen synthase (*a* + *b* form) in foetal rat liver rises 4–6-fold during the last quarter of the gestation (Ballard & Oliver, 1963; Jacquot & Kretchmer, 1964; Eisen *et al.*, 1973; Schwartz & Rall, 1973; Devos & Hers, 1974; Pines *et al.*, 1975; Watts & Gain, 1976) in a glucocorticoid-dependent way (Jacquot & Kretchmer, 1964; Plas & Jacquot, 1967). However, the question remains whether induction of 'total' glycogen synthase is the complete explanation for the glucocorticoid effect. Indeed, glycogen synthesis in foetal, as in adult, liver appears to be catalysed exclusively by the dephosphorylated (*a*) form of glycogen synthase (Eisen *et al.*, 1973; Devos & Hers, 1974; Pines *et al.*, 1975). In the developing foetal liver, the amounts of glycogen and of synthase *a* start to rise when the activity of glycogen synthase phosphatase appears (Devos & Hers, 1974). We have therefore hypothesized that glycogen synthase phosphatase may also be induced by glucocorticoids (Stalmans & Laloux, 1979).

The present paper describes how this hypothesis has been checked. It also reports a reinvestigation of synthase phosphatase in the foetal liver, in the light of recent findings on the adult enzyme (Doperé *et al.*, 1980). Some of these results have been published in a preliminary form (Vanstapel & Stalmans, 1978; Vanstapel *et al.*, 1979).

Experimental

Materials

Cortisol acetate and cortisol sodium succinate (Roussel, Brussels, Belgium) and dexamethasone sodium phosphate (Organon, Oss, The Netherlands) were commercial pharmacological preparations. Sodium pentobarbital was purchased from Serva (Heidelberg, Germany). Amyloglucosidase was from Boehringer (Mannheim, Germany), and glucose oxidase (type II) and peroxidase (type I) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The sources of other reagents, biochemicals and radiochemicals are described by Doperé *et al.* (1980). Glassine-powder paper was obtained from Eli Lilly and Co. (Indianapolis, IN, U.S.A.), and nylon mesh (210μm) was from Henry Simon Ltd. (Stockport, Cheshire, U.K.).

Handling of animals and livers

Female Wistar rats weighing about 250g were caged overnight with a male. When a copulation plug was present, the common assumption was

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made that fertilization had taken place around midnight, which marks the start of day 0 of gestation (Jost & Picon, 1970). Normal delivery takes place on day 22. For analysis of the foetal livers, the following procedure was adopted in all experiments except those with glucocorticoid-deficient foetuses (Fig. 2). Between 10:00 and 11:30h on the indicated day of gestation, the rats were anaesthetized by the intraperitoneal administration of pentobarbital (10mg/100g). About 5min later, the abdomen was opened and the foetuses were sequentially delivered by Caesarean section. The foetal livers were at once removed and swirled in ice-cold iso-osmotic saline (0.9% NaCl) to remove an extracellular glycogenolytic contaminant (Watts & Gain, 1976). Five to nine foetal livers from a single pregnant rat were pooled, dried by pulling gently over glassine paper until they became sticky, and weighed. They were homogenized manually in a Dounce tube fitted with a tight 'B' pestle in 4vol. of an ice-cold solution containing 0.25M-sucrose, 1mM-dithiothreitol and 50mM-imidazole, adjusted to pH7.4 at room temperature. The homogenates were filtered through a sheet of nylon mesh, and the filtrates used as such for the analyses described below. On each experimental day a liver sample was also taken from one or two mother animals, to obtain 'adult' reference values. Glucocorticoid-deficient foetuses were obtained and handled as described in the legend to Fig. 2.

Analyses of liver homogenates

If several assays were carried out on the same homogenates, they were done in the following order: immediately after the preparation of each homogenate, the determination of glycogen was initiated; the assays of glycogen synthase were started at once after the preparation of the last homogenate, and those of glycogen synthase phosphatase within 2h. Glycogen was determined as described by Chan & Exton (1976), with enzymic determination of glucose (Dahlqvist, 1961) as the final step. Glycogen synthase *a* and 'total' enzyme (*a* + *b* form) were measured at 25°C as described (Doperé *et al.*, 1980). One unit of glycogen synthase converts 1μmol of substrate into product/min under these assay conditions.

The activity of glycogen synthase phosphatase was determined from the rate at which the enzyme converted purified glycogen synthase *b* into *a* at 25°C. All homogenates were assayed at a final concentration of 2% (w/v). The substrates were different subfractions of glycogen synthase *b*, isolated from dog liver and termed b_1, b_2 and b_3 (Doperé *et al.*, 1980). The cell fractions used in part of the present study (see Fig. 4) were prepared from adult rat liver as described by Doperé *et al.* (1980): an isolated protein–glycogen complex served as 'G-component', and a post-glycogen supernatant as a crude source of 'S-component'. In the synthase phosphatase assay these cell fractions were adjusted to a final concentration equivalent to that of a 2% (w/v) liver homogenate.

Results and discussion

Normal developmental patterns

As a prerequisite to the main objective of this study, the temporal relationship between glycogen deposition and the development of key enzymes in the foetal rat liver was re-examined. The crucial period may be appreciated from Fig. 1. Significant increases in all parameters examined are first observed on day 18, in agreement with previous observations (see the introduction). It is important to note that on day 17, i.e. before glycogen synthesis occurs, the amount of 'total' glycogen synthase is already one-third of the adult value. The concentration of synthase *a*, however, is then negligible. The general pattern is thus in agreement with the proposal (Devos & Hers, 1974) that the appearance of glycogen synthase phosphatase initiates, by way of the activation of glycogen synthase, the process of glycogen deposition in the foetal liver.

Effects of glucocorticoids

Two approaches *in vivo* were used in the present study to delineate the role of glucocorticoid hormones in the induction of specific enzymes. In one series of experiments the effects of prematurely administered steroids on glycogen metabolism were explored. In a second approach, we have examined the changes caused by glucocorticoid deficiency and by the substitutive administration of cortisol.

The aim of the first approach was to obtain a premature accumulation of glycogen in the foetal liver by the early administration of glucocorticoids; in that condition any relevant enzyme should be induced precociously as well. The injection of steroids to the mother was chosen, rather than the established but tedious technique of foetal injection through laparotomy. However, a single dose of cortisol (50mg as sodium succinate plus 25mg as acetate), administered at various stages of the gestation, failed to produce within 1day a significant change in the amount of glycogen or in the activities of glycogen synthase and of synthase phosphatase. Wishart & Dutton (1977) have described that, in contrast with a poor effect of cortisol, the injection of dexamethasone to the mother rat resulted in a rapid dose-dependent induction of UDP-glucuronyltransferase in the foetal liver. As shown in Fig. 1, dexamethasone treatment of gravid rats, starting on day 15, had increased already on day 16 all parameters examined (glycogen content; concentration of total glycogen synthase and of syn-

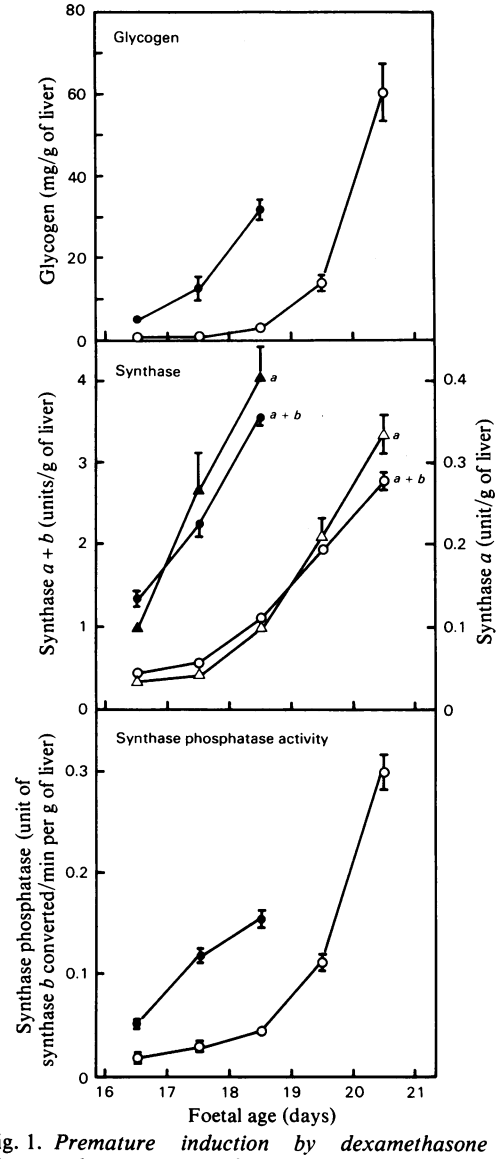

Fig. 1. *Premature induction by dexamethasone of glycogen deposition and of key enzymes in the liver of the developing foetal rat*

Gravid rats were injected intraperitoneally at 09:30h on days 15, 16 and 17, either (●, ▲) with 0.5mg of dexamethasone sodium phosphate (0.38mg of steroid) or with an equal volume of iso-osmotic saline (○, △). Synthase phosphatase was measured with synthase b_2 as substrate. Each point shows the mean of observations on three or four litters. Vertical bars, indicated where the scale permits, represent ±S.E.M. The relevant adult values from saline-treated animals were as follows (mean ±S.E.M. for 7 livers): glycogen synthase $a+b$, 1.64±0.13units/g of liver; synthase phosphatase, 0.56±0.02unit of synthase b_2 converted/min per g of liver. Statistical analysis of steroid-treated versus control by Student's t test: $P<0.01$ for each parameter on each gestational day.

thase a; and activity of glycogen synthase phosphatase) to at least the value observed in control foetuses on day 18. On the ensuing days the foetuses of steroid-treated mothers kept a similar lead over their untreated counterparts.

After adrenalectomy of the mother, glycogen accumulates normally in the foetal rats' liver; individual foetuses in such a litter can be rendered completely glucocorticoid-deficient by decapitation, which prevents the development of the adrenal cortex. The specificity of the latter effect can be evaluated by a substitutive treatment of some decapitated foetuses with cortisol (Jacquot, 1959; Jost & Picon, 1970). We performed the decapitation on day 17 *post coitum*, and on day 20 three groups of foetuses were examined (Fig. 2). Although the number of observations on decapitated foetuses remained small because of a considerable (75%) foetal mortality, clear conclusions can be reached. The decapitated foetuses accumulated 13 times less hepatic glycogen than did their control littermates, and the activity of total glycogen synthase remained 50% lower; effects of similar magnitude have been observed previously (Jacquot & Kretchmer, 1964; Plas & Jacquot, 1967). In decapitated foetuses the synthase phosphatase activity on day 20 remained at 25% of the control value. A substitutive treatment with cortisol restored the glycogen deposition and the activity of total glycogen synthase completely, and increased 3-fold the activity of synthase phosphatase (Fig. 2).

We conclude that glucocorticoids, which increase transiently in rat foetal plasma during days 18–20 (Holt & Oliver, 1968; Cohen, 1973; Dupouy *et al.*, 1975), induce hepatic glycogen synthesis by a dual mechanism: they provide an excess of substrate (glycogen synthase) as well as the phosphatase required to keep part of glycogen synthase in the active form. Since glucocorticoids are able to evoke these responses precociously when administered prematurely to the intact organism (Fig. 1), they meet the criteria set by Greengard & Dewey (1970) to qualify as the physiological trigger. The concentration of insulin in rat foetal plasma rises severalfold after day 18 (Felix *et al.*, 1971; Cohen & Turner, 1972; Girard *et al.*, 1974; Watts *et al.*, 1976). Insulin enhances glycogen synthesis in isolated foetal liver (for a recent discussion, see Plas *et al.*, 1979). Our observations that glycogen synthesis and the underlying enzymic machinery can be induced by dexamethasone as early as day 16 (Fig. 1) do not, however, suggest an essential role of insulin in prenatal glycogen synthesis.

Development of the two components of synthase phosphatase

While this investigation was in progress, we obtained evidence that the activity of glycogen

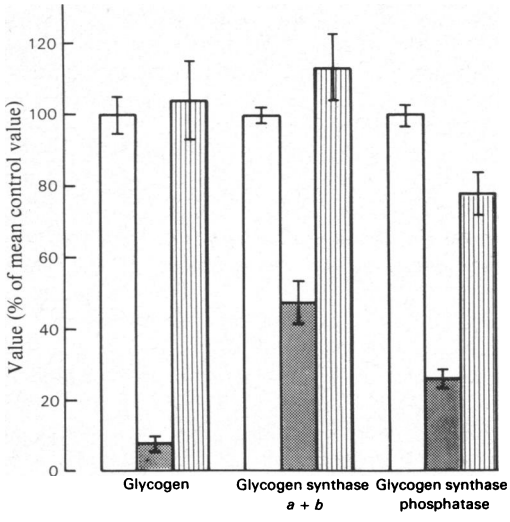

Fig. 2. *Effects of glucocorticoid deficiency and of cortisol substitution on glycogen deposition and key enzymes in foetal rat liver*

Bilateral adrenalectomy of pregnant rats was performed on day 13 or 14. On day 17 two foetuses of each litter were decapitated *in utero* (Jacquot, 1959). One of these was injected subcutaneously with a suspension of cortisol acetate (0.6 mg in 25 μl), and the other received 25 μl of iso-osmotic saline. On day 20, the surviving foetuses were handled as in the Experimental section, except for the preparation of homogenates from individual foetal livers. Results are given as means ± S.E.M. for the indicated number of observations. Values for the 100% 'control' values are as follows: glycogen, 52.5 mg/g of liver; glycogen synthase $a+b$, 2.29 units/g of liver; activity of glycogen synthase phosphatase, measured on an unseparated mixture of synthases b_2 and b_3, 0.18 unit of synthase b converted/min per g of liver. Student's t test indicates a significant effect ($P<0.001$) of decapitation and of cortisol substitution on each parameter. □, Control ($n=17$); ▩, decapitated ($n=5$); ▥, decapitated + cortisol ($n=3$).

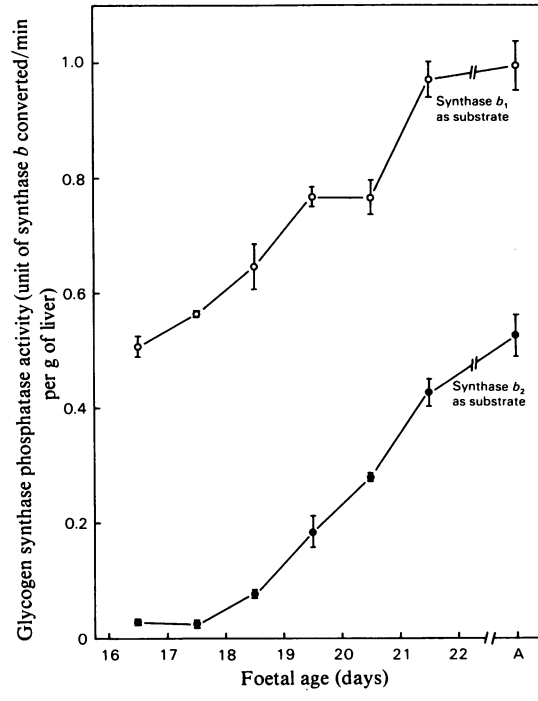

Fig. 3. *Effect of substrate on the expression of synthase phosphatase activity in the foetal rat liver*

Results are shown as means ± S.E.M. for observations on three litters and on 11 adult livers (A). For further details see the text.

synthase phosphatase in the adult liver stems from two protein components: a cytosolic 'S-component' and a glycogen-bound 'G-component' (Doperé *et al.*, 1980). Distinct types of synthase b that can be isolated from dog liver show different substrate properties: the activation of synthases b_2 and b_3, which predominate in the liver, requires both components of synthase phosphatase; synthase b_1, however, is efficiently activated by the S-component alone, and only some 35% faster in the presence of both components. We report here investigations on the possibility that the manyfold increase in synthase phosphatase activity during late gestation (Fig. 1) might be caused by the specific development of one component only.

In a first approach, foetal liver homogenates were examined for their ability to activate different types of hepatic synthase b. When synthase b_2 served as substrate, synthase phosphatase activity on days 16–17 of gestation was about 5% of that measured in the liver of the mother animal; it increased progressively thereafter, and near term reached 80% of the adult value (Fig. 3). The same pattern was observed in earlier experiments with an unseparated mixture of synthases b_2 and b_3 (results not shown). In contrast, synthase phosphatase activity measured on synthase b_1 amounted on day 16 already to 50% of the adult value, and it only doubled during the ensuing days (Fig. 3).

Next the activity of synthase phosphatase in foetal liver homogenates was determined on synthase b_2, both as such and in the presence of added cell fractions containing specifically the S- or the G-component (Fig. 4). The reasoning behind the experiment was that, if the development of the two components was not synchronous, then the addition of the 'missing component' early in the gestation would produce an unexpectedly high increase in

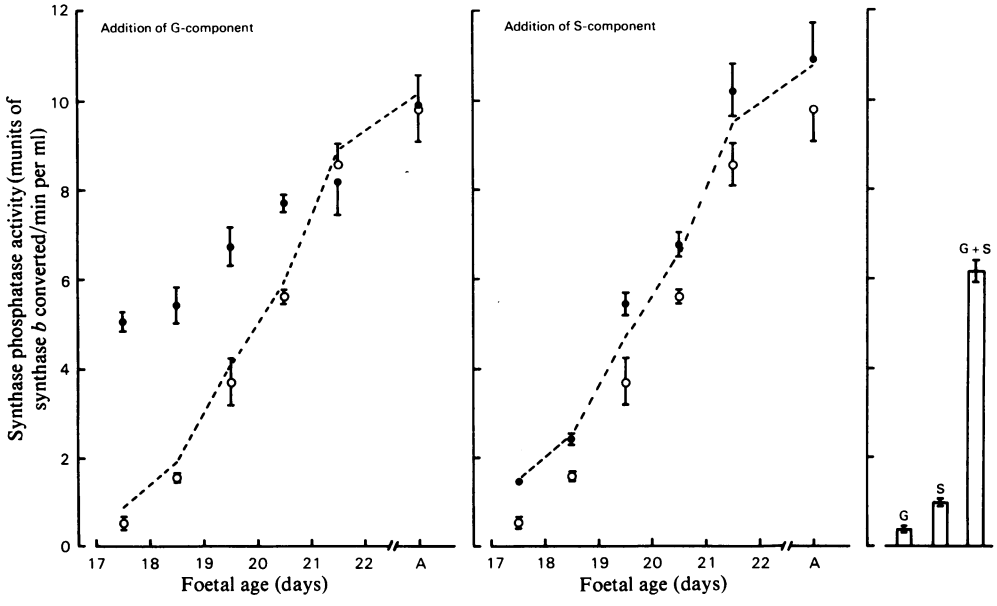

Fig. 4. *Effect of added G-component or S-component on synthase phosphatase activity in liver homogenates from foetal or adult rats*

Left and central panels: synthase phosphatase activity was measured in assay mixtures containing glycogen synthase b_2 and 2% (w/v) liver homogenates, without (○) or with (●) a preparation of G-component or S-component as indicated. The broken line indicates the activity that would result from simple addition of the activities measured separately. Results are shown as means ± S.E.M. for observations on three litters and on six adult livers (A). Right-hand panel: synthase phosphatase activity of the preparations of G- and S-component, incubated separately or combined; vertical bars are ± S.E.M. for three independent determinations.

synthase phosphatase activity. This approach appears adequate, since the activity of synthase phosphatase in homogenates of adult liver is not stimulated by the addition of either component (Fig. 4). Added S-component produced only an additive effect in foetal liver homogenates. In contrast, addition of the G-component to a foetal liver homogenate on day 17 stimulated the synthase phosphatase activity about 10-fold. During the ensuing days this stimulation decreased while the activity of synthase phosphatase developed progressively.

These two approaches (Figs. 3 and 4) indicate that the S-component of synthase phosphatase is largely or completely developed before glycogen synthesis occurs. The manyfold increase in the activity of synthase phosphatase during late gestation appears then to be caused by a glucocorticoid-induced synthesis of the G-component. Further research must answer the important question of how the G-component actually works.

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