PROLIFERATION OF ENDOPLASMIC RETICULUM WITH ITS ENZYME, UDP-GLUCURONYLTRANSFERASE, IN CHICK EMBRYO LIVER DURING CULTURE

Effects of Phenobarbital

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ABSTRACT

UDP-Glucuronyltransferase (GT) activity increases in chick embryo liver during culture from zero to a steady-state level at or above adult values. The GT activity (*o*-aminophenol as acceptor) is located entirely in the membranes of the endoplasmic reticulum (ER) and the question arises whether ER increases along with GT. Earlier work showed that the synthesis and degradation rates of GT can be varied in culture over wide ranges by choosing embryo livers of different ages and with phenobarbital. In the present study we measured the GT activities and the concentrations of ER (using stereologic methods) in 5- and 11-day embryo liver during culture with and without phenobarbital. We found that GT and ER always increased in a constant ratio of 2.2×10^{-9} U of GT activity per square micrometer of membrane, suggesting that the synthesis and degradation of GT are coupled to the synthesis and degradation of ER. A general structure for ER is proposed to explain this finding.

Glucuronides are synthesized in the liver by UDP-glucuronyltransferase (GT) (EC 2.4.1.17). In mammals, GT activity is absent in the fetus (8) and increases to adult values around the time of birth (16). Similarly in the chick, GT activity is absent or very low in embryo liver and rapidly increases to adult values on hatching (9). GT activity is localized entirely in the membranes of the smooth endoplasmic reticulum (SER) and rough endoplasmic reticulum (RER) (7, 10, 12, 19). The question arises whether development of GT activity is associated with proliferation of SER and RER (2, 4).

Earlier work showed that GT activity increases in chick embryo liver during cell or organ culture from zero to a steady-state level at or above adult values, and is probably directly related to the amount of enzyme (4). Younger tissues develop GT activity more rapidly and to a higher level than older tissues and can be further stimulated by phenobarbital. Therefore, the synthesis rate (S) and degradation rate constant *(K)* of GT (calculated from the kinetic data) can be varied in culture by choosing embrto livers of different ages and with phenobarbital. In 5- and 11-day embryo liver cultured with and without phenobarbital, the S of GT varies over a 10-fold range, the *K* by a factor of 1.7 (4). In the present study, using stereologic methods on electron micrographs, we measured the concentrations of SER and RER

(along with GT activity) in these same tissues. We found that GT and ER (SER plus RER) always increased during culture in a constant ratio of 2.2 $\times 10^{-9}$ U GT activity per square micrometer of membrane. Thus, the S and the K of GT varied with embryo age and phenobarbital in a constant ratio to the S and K of ER, suggesting that the synthesis and degradation of GT is coupled to the synthesis and degradation of ER. A general structure for ER is proposed to explain this finding.

Our observations also support the view that new membrane (with enzyme) is made in the RER which is subsequently converted to SER and that there is an equilibrium between SER and RER which may flow in either direction.

MATERIALS AND METHODS

Fertile eggs (White Leghorn) were obtained from George F. Shaw Inc., West Chester, Pa. The eggs were maintained in a standard egg incubator. Embryos *in ovo* were treated with phenobarbital by placing 1 ml of a sterile solution of sodium phenobarbital (1 or 10 mg per milliliter, adjusted to pH 7.1 with HCl) into the egg air space. The extent of phenobarbital absorption into the embryo from the air space was not determined, and was probably different at various ages. However, the drug was detectable by standard methods (3) in embryo blood and tissues.

Tissue was cultured on Millipore filter rafts (Millipore Corp., Bedford, Mass.) over Eagle's medium, with and without phenobarbital (5.5 mM), as described previously (4, 18). A raft bore five whole 5-day embryo livers (approx. 1 mg per liver), or similar size fragments of 11-day embryo liver, each from a different embryo. Some tissues were prepared for electron microscopy, others for assay of GT activity. The method for assaying GT activity (o-aminophenol as acceptor) has been described elsewhere (4, 18); the μg o o-aminophenylglucuronide formed in the assay system in 30 min per milligram tissue protein was converted into units of GT activity per cubic micrometer of cytoplasm. A unit of GT activity was defined as making 1 μg product (o-aminophenyl glucuronide) per hour. 1 mg of embryo liver contains 0.1 mg of protein. 1 mg of tissue has a vol of approx. 1 mm^3 or 10^9 μm^3, which is 80% cytoplasm. Therefore, GT activity expressed as microgram of o-aminophenylglucuronide formed in 30 min per milligram of protein when multiplied by $2 \times 0.1 \times 1.25 \times 10^{-9}$ equals the units of GT activity per cubic micrometer of cytoplasm.

Fresh and cultured tissues were prepared for electron microscopy by placing small fragments (less than 1 mg) into a glutaraldehyde-paraformaldehyde solution (11) for 25 min at room temperature (2.5% and 2% by vol, respectively, in 0.1 M sodium cacodylate and 2 mM $CaCl_2$ at pH 7.1). The fragments of tissue were then washed three times in a glucose solution at 0°C (0.25 M glucose, 0.1 M sodium cacodylate, 2 mM $CaCl_2$ at pH 7.1) and placed in an OsO_4 solution at 0°C for 30 min (1% OsO_4, 0.1 M sodium cacodylate, 2 mM $CaCl_2$, at pH 7.1), washed again, dehydrated in a graded series of ethanol solutions to 100% at 0°C, placed in a propylene oxide solution and embedded in a plastic mixture of Epon 812 and Araldite 502 (15). Thin sections, with gray to pale gold interference colors, were cut and placed on 150-mesh copper grids, stained with uranyl acetate, and then lead acetate. Glycogen was only lightly stained so as not to obscure SER.

Observations were made with the JEOL 120B electron microscope. After initial focusing, fields selected at random without viewing were photographed on Kodak EM film 4489 (Eastman Kodak Co., Rochester, N.Y.). Prints were enlarged to 249 × 200 mm at a magnification of 20,000. Grids of horizontal and vertical lines, 25 mm apart, were marked on the prints. The total length of lines on a print was approx. 4,000 mm. The grid lines were used (see Loud [13] for detailed method) to measure: *(a)* the percent volume of liver cell cytoplasm occupied by mitochondria, lipid droplets, and microbodies, and *(b)* the square micrometers of SER and RER per cubic micrometer of cytoplasm.

$$\text{Percent vol of cytoplasm occupied by structure} = (L)/(L_{total}) \times 100, \quad (1)$$

where L is the length of grid lines superimposed upon structure, e.g., mitochondria, and L_{total} is the length of grid lines superimposed upon cytoplasm.

$$\text{Square micrometers of SER and RER per cubic micrometer of cytoplasm} = 2CM/1{,}000L, \quad (2)$$

where C is the number of crossings of grid lines over membrane edge, L is the number of millimeters of grid lines over cytoplasm, and M = magnification. Each point in the figures represents the means of the separate analyses of 10 electron micrographs. The number of crossings to determine the square micrometers of SER or RER per cubic micrometer of cytoplasm varied between 600 and 8,000, except in the normal 4- to 6-day old embryo liver *in ovo*, in which the number of crossings over SER (Fig. 1) was less than 600. Thus, the counting errors (13) were almost always less than 5%.

RESULTS

Embryo Liver In Ovo

GROWTH: Chick embryo liver was studied *in ovo* to provide a base line for the culture studies. Embryo liver (by vol) is made up almost entirely of parenchymal cells. It increases in weight from approx. 0.4 mg on day 4 of egg incubation to 1 g at hatching (17). The growth rate of embryo liver is highest shortly after its appearance early on day

3 of egg incubation, and steadily declines with age, more rapidly before day 5 than afterwards. Mitotic activity decreases similarly (14). Liver cell volume approx. doubles between day 3 and 5 and remains essentially unchanged thereafter (6). Thus, before day 5, liver growth is accounted for by increases in parenchymal cell size and number, after day 5, solely by an increase in cell number. Injection of 1 mg of sodium phenobarbital into the air space of fertile eggs did not significantly affect liver weight. Hatching time and embryo survival were also unaffected.

ULTRASTRUCTURE: Electron micrographs of chick embryo livers obtained at various stages of development (day 4–16 of egg incubation) were analyzed by stereologic methods to determine the square micrometers of SER and RER per cubic micrometer of liver cell cytoplasm (Figs. 1 and 2) and the percent volume of cytoplasm occupied by mitochondria (Fig. 3), microbodies, and lipid droplets (see next paragraph). In addition, embryo livers were analyzed after administration of phenobarbital to the embryo. The predominant effect of phenobarbital was to increase the concentration of SER (Fig. 1). The incidence of membrane connections between SER and RER was also greatly increased, suggesting that SER arises from RER at these connections. During the initial 2 days after phenobarbital injection, membrane connections between SER and RER were observed in circumscribed areas between small patches of SER and the edges of stacked RER cisterns. Later, the patches of SER enlarged. The enlarged patches of SER were far fewer in number than the small patches, suggesting that either some fusion or regression had occurred. Phenobarbital had little or no effect on the concentration of RER (Fig. 2). Thus, under the influence of phenobarbital, new membrane is probably synthesized in RER and an equivalent amount converted to SER.

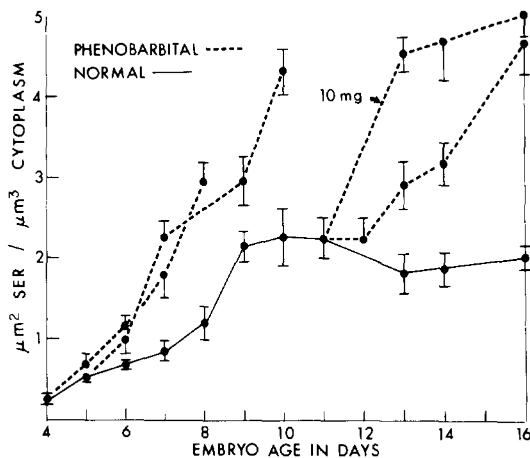

FIGURE 1 Square micrometers of SER per cubic micrometer of cytoplasm in chick embryo liver cells from day 4 to day 16 of egg incubation. Solid line indicates normal development. Broken lines indicates development after single injection of 1 mg sodium phenobarbital into eggs on day 3, day 5, or day 11 of incubation; 10 mg dose also tested on 11 day eggs. Points represent the means of the separate analyses of 10 electron micrographs. The SEM's are indicated by vertical bars. Tissue from one embryo was used to make three or four micrographs. Glycogen was first observed in occasional cells on day 7; it was observed in most cells by day 9.

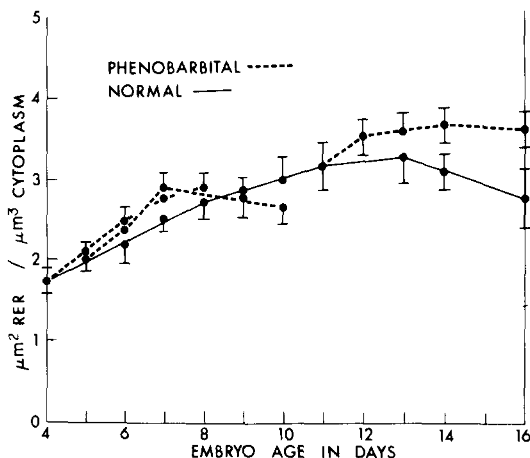

FIGURE 2 Square micrometers of RER per cubic micrometer cytoplasm in chick embryo liver cells from day 4 to day 16 of egg incubation. Solid line indicates normal development. Broken lines indicate development after single injection of 1 mg of sodium phenobarbital into eggs on day 3, day 5, or day 11 of incubation: 10 mg dose also tested on 11 day eggs but because the values were virtually the same as after the 1 mg dose, results were averaged. Points represent the means of the separate analyses of 10 electron micrographs (20 for 11 day liver after phenobarbital administration). The SEM's are indicated by the vertical bars. Tissue from one embryo was used to make three or four micrographs.

The percent volume of cytoplasm occupied by mitochondria initially increases, then decreases during embryonic development. Phenobarbital has no effect (Fig. 3). The percent volume of cytoplasm occupied by microbodies increases from 0.5% on day 4 of egg incubation to a plateau of 2.5% on day 9. Lipid droplets gradually increase from 0.2% on day 4, to 1% on day 11, to 4% on

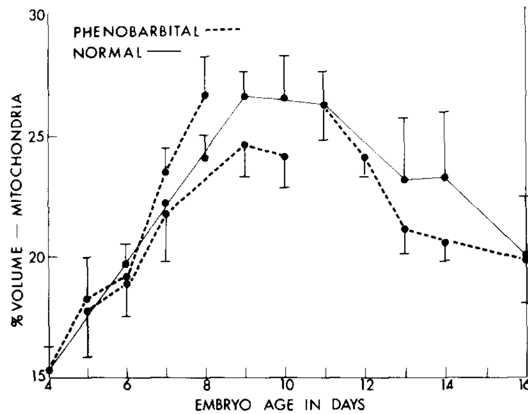

FIGURE 3 Percent vol of cytoplasm occupied by mitochondria in chick embryo liver cells from day 4 to day 16 of egg incubation. Solid line indicates normal development. Broken lines indicate development after single injection of 1 mg sodium phenobarbital into eggs on day 3, day 5, and day 11 of incubation. Points represent the means of the separate analyses of 10 electron micrographs. The SEM's are indicated by vertical bars. Tissue from one embryo was used to make three or four micrographs.

day 16 (SEMs approx. 15% of values). Phenobarbital decreases these values, more markedly and for a longer time in the younger embryos.

Embryo Liver During Culture

VIABILITY: Fragments of 5-day old embryo liver explanted onto Millipore filter rafts over Eagle's medium lose approx. 50% of their protein and DNA during 5 days of culture. Phenobarbital in the medium decreased these losses to 30%. The loss of protein and DNA is probably the result of cell death and rapid dissolution because no necrosis is apparent, and the ratio of DNA to protein (wt/wt) remains constant (4). Fragments of 11-day old embryo liver during 5 days of culture maintain a constant protein and DNA content, which is unaffected by phenobarbital. There is little or no cell proliferation in these cultures (18). Cell types other than parenchymal cells occupy only a few percent of the tissue volume (4). The percentage of protein in chick embryo liver is between 10% and 13% by weight (17), and does not change during culture (18).

ULTRASTRUCTURE: Livers from 5- and 11-day old embryos, except for their ER, go through essentially the same ultrastructural changes during 5 days of culture. The percent volumes of cytoplasm occupied by lipid droplets and microbodies fall to approx. 50% of the starting values within the first two days of culture and remain there. The percent volume of mitochondria stays at starting values through the culture period. Phenobarbital has no effect on these changes, which appear to be characteristic of culture rather than normal development.

In 5-day embryo liver during culture, the concentration of RER increases to higher than normal developmental values, more rapidly with phenobarbital in the medium, but to the same plateau as without the drug (Fig. 4). No SER develops unless phenobarbital is added to the medium. However, even with phenobarbital in the medium, only a small amount of SER appears.

In 11-day old embryo liver during culture, the concentration of RER increases and SER decreases (Fig. 4). Only very occasional autophagic vacuoles and essentially no degeneration are present to indicate possible destruction of SER, so we wish to suggest that the SER and RER are in equilibrium and that we are observing a flow of membrane from SER to RER. There is a small net increase in total ER (RER plus SER), presumably the result of synthesis (Fig. 5, upper). The inference that SER flows into RER is strongly supported by the finding, described below under GT development, that the net increases in total ER

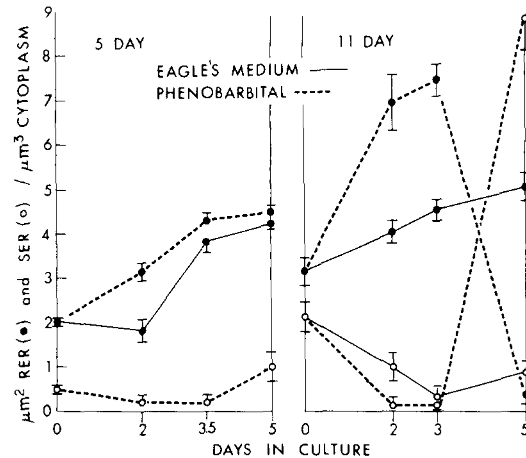

FIGURE 4 Square micrometers of RER (●) and SER (O) per cubic micrometer of cytoplasm in 5- and 11-day old chick embryo liver after organ culture on rafts over Eagle's medium with (broken lines) and without (solid lines) 5.5 mM phenobarbital. Points represent the means of the separate analyses of 10 electron micrographs. The SEM's are indicated by vertical bars. Tissue from one embryo provided three or four micrographs.

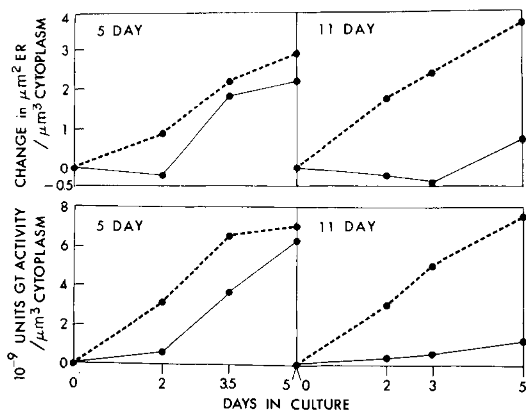

FIGURE 5 Change in the square micrometer of ER (SER plus RER) per cubic micrometer of cytoplasm in 5- and 11-day old chick embryo liver after organ culture (upper). Values were calculated from Fig. 4 by adding the areas of SER and RER per cubic micrometer cytoplasm after various culture periods, and substracting the values at the beginning of culture. UDP-glucuronyltransferase *(GT)* activity per cubic micrometer of cytoplasm in 5- and 11-day embryo liver after organ culture (lower). A unit of activity forms 1 μg *o*-aminophenylglucuronide per hour. Points represent the means of separate determinations on three culture dishes; a culture dish contained five whole 5-day embryo livers, or similar size fragments of 11-day embryo liver, each from a different embryo. The means were within 10% of the values obtained earlier (reference 4) on a larger number of determinations (10 culture dishes per point). Solid lines indicate tissues maintained on rafts over Eagle's medium. Broken lines indicate 5.5 mM phenobarbital in medium.

proceed in constant proportion to the increases in GT (Fig. 6).

Phenobarbital added to medium accelerates the increase in RER and the decrease in SER (Fig. 4) observed in 11-day embryo liver during culture. The net increase in total ER is much greater with phenobarbital in the medium than without (Fig. 5, upper). After 3 days of culture with phenobarbital, the equilibrium between RER and SER dramatically shifts and a rapid and almost complete conversion of RER to SER takes place (Fig. 4). Thus, we suggest that two processes affect the concentrations of SER and RER in cultured 11-day embryo liver: *(a)* synthesis of RER, and *(b)* an equilibrium between SER and RER, which, during culture, at first moves toward RER and then, later, if phenobarbital is present, shifts toward SER (Figs. 4 and 5). Free ribosomes markedly decrease in chick embryo liver during culture with or without phenobarbital in the medium.

Differences between 5- and 11-day embryo liver as regards changes in SER and RER during culture (Fig. 4) are undoubtedly related to the limited capacity of 5-day embryo liver to form SER (Fig. 1) and the failure of the tissue to develop this ability (Fig. 4) to a significant extent during culture (1, 2).

GT DEVELOPMENT: The pattern of GT development in 5- and 11-day old embryo liver during culture in the presence and absence of phenobarbital has been described previously (4). Part of this work was repeated here (Fig. 5, lower) along with measurements of ER (Fig. 5, upper). GT and total ER increased in a constant ratio of 2.2 ± 0.2 (SEM) × 10^{-9} U GT activity per square micrometer of membrane, as can be seen by superimposing the upper and lower graphs in Fig. 5 (also see Fig. 6).

New membrane and enzyme are presumably synthesized in the RER which then may flow into

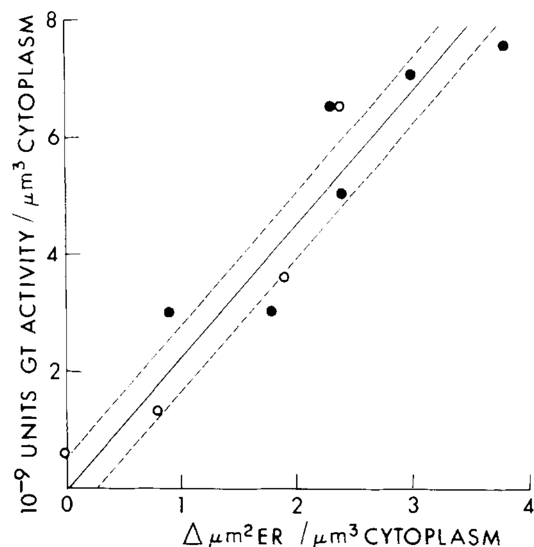

FIGURE 6 UDP-glucuronyltransferase *(GT)* activity plotted against the change (Δ) in square micrometers of ER in chick embryo livers during organ culture. Values were taken from Fig. 5, upper and lower; omitted were the initial two values on 11-day embryo liver cultured without phenobarbital, which were within 1 SD of the origin; also omitted were the two starting values, which were at the origin. Solid symbols indicate 5- or 11-day embryo liver maintained on rafts over Eagle's medium containing phenobarbital. Open symbols indicate no phenobarbital in medium. Solid line indicates average value of GT activity per square micrometer of additional ER. A unit of GT activity produces 1 μg *o*-aminophenylglucuronide per h. Broken lines represent ±1 SD.

SER. This sequence is suggested by observations in both 5- and 11-day embryo liver during culture. In a 5-day embryo liver, little or no SER appears (Fig. 4), so that new membrane and new enzyme must be localized entirely in RER. In 11-day embryo liver during the initial 3 days of culture with phenobarbital, SER almost completely disappears as RER increases (Fig. 4). After 3 days of culture, RER falls almost to zero as an equivalent amount of SER appears. Because GT is present exclusively in the membranes of the ER throughout culture, new enzyme must initially have appeared in the RER which is then converted to SER.

DISCUSSION

Earlier work showed GT increases in chick embryo liver cells during culture from zero to a steady-state level at or above adult values. The synthesis rates (S) of GT differ over a 10-fold range in 5- and 11-day embryo liver cultured with and without phenobarbital. The degradation rate constants (K) differ by a factor of 1.7 (4). The lowest S and K are observed in 11-day embryo liver cultured with Eagle's medium alone, and the highest in 5-day embryo liver cultured with Eagle's medium and phenobarbital (4). In the present study, using stereologic methods, we measured the concentrations of SER and RER, along with GT activity, in these same tissues. We found that GT and ER (SER plus RER) always increased during culture in a constant ratio of 2.2×10^{-9} U of GT activity per square micrometer of membrane (Fig. 6). Thus, the S and the K of GT varied with embryo age and phenobarbital in a constant ratio to the S and K of ER, suggesting that the synthesis and degradation of GT are coupled to the synthesis and degradation of ER. Also, considering that GT is localized in ER, GT is probably uniformly distributed in new ER.

Current models of plasma membrane have globular proteins freely diffusing in a lipid bilayer (18). Trump et al., however, have shown that ER, unlike plasma membrane, retains its form after lipid extraction, and that the lipid components can be reintroduced to restore lost enzyme activities (20). Therefore, ER appears to have a rigid protein framework. We suggest that the protein framework has a constant composition of which GT is a part and that the framework bears a constant relationship to membrane area. This would explain the coupling of GT and membrane synthesis (area). For the same reason, the degradation of GT and that of membrane occur together.

Many earlier in vivo studies have shown that the enzyme composition of ER changes with development, and also during physiological adjustment to drugs, fasting, etc. (5). This would argue against the idea that the protein composition of ER is fixed. However, the relatively simple and constant environment of culture may result in the synthesis of a constant membrane, or at least a membrane with a constant protein framework, while the varying and complex milieu within the organism results in the synthesis of a series of different membranes, producing a mosaic, and giving a picture of variable composition. The idea that GT is an integral part of a protein framework in the membrane is compatible with the failure of many attempts to solubilize and purify GT (7).

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