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grande quantité de matière amorphe déposée dans des endroits circonscrits du cytoplasme. Il paraît que la matière amorphe est déposée au début dans la cavité du réticulum endoplasmique rugueux qui dilate et les membranes se brisent en morceaux pour libérer la matière amorphe dans le cytoplasme. Les hépatocytes du poussin de 5 jours produisent beaucoup plus de matière amorphe que les cellules de 11 jours, qui ont en formé si peu. Si, comme beaucoup d'auteurs ont suggéré (Banjo & Nemeth, 1976; Ernster & Orrenius, 1965; Orrenius, 1965; Orrenius, Ericsson & Ernster, 1965), quelques-uns des enzymes produits par suite de la stimulation des cellules par la phénobarbitale sont inséparablement fixés sur les membranes du réticulum endoplasmique (RER et SER), c'est-à-dire que les enzymes sont stables seulement quand ils s'attachent à la membrane, il est probable que la grande quantité d'enzymes produite par le réticulum endoplasmique hypertrophié et rugueux (Banjo & Nemeth, 1976) dans les hépatocytes de 5 jours en culture sous l'influence de la phénobarbitale n'ont pas pu trouver assez de membrane du réticulum endoplasmique lisse pour s'y attacher dans les mêmes cellules de 5 jours dont la capacité pour produire du réticulum endoplasmique lisse est très restrainte. Donc, cette grande quantité d'enzymes et les membranes peu nombreuses ne sont pas organisées et sont par conséquent libérées comme la matière amorphe dans le cytoplasme. D'autre part, les hépatocytes de 11 jours ont formé très peu de matière amorphe, ce qui résulte probablement de leur capacité élevée de produire les membranes du réticulum endoplasmique lisse. On suggère que la grande quantité de matière amorphe libérée dans les cellules de 5 jours représente les enzymes et membranes non organisées.

Introduction

The normal response of liver cells *in ovo* and *in vitro* to phenobarbital has been described by several authors. (Banjo & Nemeth, 1976; Ernster & Orrenius, 1965; Essner & Novikoff, 1963; Herman, Eber & Fitzgerald, 1962; Jones & Armstrong, 1965; Orrenius, 1965, Orrenius *et al.*, 1965; Remmer & Merker, 1965). This consists of initial proliferation of the rough endoplasmic reticulum (Banjo & Nemeth, 1976) on which the drug-metabolizing enzymes are also synthesized (Ernster & Orrenius, 1965). The rough endoplasmic reticulum proliferation is followed by the establishment of RER → SER conversion centres (Banjo & Nemeth, 1976), where the rough endoplasmic reticulum sheds its ribosomes and the ribosome-denuded membrane becomes the smooth endoplasmic reticulum which now contains the same enzymes (Ernster & Orrenius, 1965). Other authors (Remmer & Merker, 1965) observed only the proliferation of the smooth endoplasmic reticulum. This is because the observation was made at the last stage of the response.

Abnormal response of liver cells have been observed following the administration of toxic substances which are carcinogenic to the liver (Emmelot & Benedetti, 1960; Emmelot & Benedetti, 1961; Herman *et al.*, 1962; Mikita & Luse, 1964; Rouiller & Simon, 1962; Solomon, 1962; Simms & Saunders, 1942; Steiner & Baglio, 1963; Steiner, Miyai & Phillips, 1964), or high doses of sodium phenobarbital (Gnosspelius & Orrenius, 1967). This abnormal response consists of the formation of concentric whorls of closely packed smooth membranes which have been referred to as finger-prints (Emmelot & Benedetti, 1960; Steiner & Baglio, 1963), Nebenkern (Emmelot & Benedetti, 1961; Rouiller & Simon, 1962) and myelin figures (Rouiller & Simon, 1962.

This paper describes an entirely new response of the chick embryo liver cells when treated with phenobarbital in culture.

Materials and methods

The organ culture was set up as previously described (Banjo & Nemeth, 1976). Tiny pieces of liver from 5-day-old chick embryos were cultured on millipore filter rafts (Millipore Corp., U.S.A.) which is supported by nitex nylon net over Eagle's medium with Earl's salt and antibiotic/antimycotic mixture with and without phenobarbital (5.5 mmol). Tissues from five 5-day-old and five 11-day-old embryos were cultured on separate dishes. The experiment was set up such that the liver from each embryo is contained in the three sets of culture. The dishes were incubated in air:CO_2 mixture at 19:1 v/v at

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THE PRODUCTION OF SMOOTH ENDOPLASMIC RETICULUM AND FORMATION OF AN ABNORMAL AMORPHOUS MATERIAL BY THE IMMATURE 5-DAY-OLD CHICK HEPATOCYTES IN CULTURE UNDER THE INFLUENCE OF PHENOBARBITAL

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Summary

The experiment was performed to see if the 5-day-old chick hepatocytes which normally do not produce smooth endoplasmic reticulum in culture, would do so under the influence of phenobarbital. The results indicated that under the influence of phenobarbital, the 5-day hepatocyte produces a small amount of smooth endoplasmic reticulum. However, a more significant observation is that the 5-day-old hepatocytes in the presence of phenobarbital also produce a large amount of amorphous material which is deposited in circumscribed areas of the cytoplasm. It appears that the amorphous material is initially deposited into the cisternal space of the rough endoplasmic reticulum which dilates and the membrane fragments to release the amorphous substance into the cytoplasm. The 5-day-old chick hepatocytes produce a lot more amorphous material than the 11-day-old cells, which formed so little. If, as suggested by many authors (Banjo & Nemeth, 1976; Ernster & Orrenius, 1965; Orrenius, 1965; Orrenius, Ericsson & Ernster, 1965, some of the enzymes produced as a result of the stimulation of the cells by phenobarbital are inseparably located on the membranes of the endoplasmic reticulum (RER and SER), i.e. the enzymes are only stable when attached to the membrane, it may be that the large amount of enzymes produced by the hypertrophied rough and endoplasmic reticulum (Banjo & Nemeth, 1976) in the 5-day-old hepatocytes in culture under the influence of phenobarbital could not find enough smooth endoplasmic reticulum membrane for attachment in the same 5-day-old cells whose capacity for producing smooth endoplasmic reticulum is very limited. Therefore, this large amount of enzyme and the few membranes are not organized and are therefore liberated, as the amorphous material, into the cytoplasm. The 11-day-old hepatocytes, on the other hand, formed very little amorphous material, and this may be due to its high capacity to make smooth endoplasmic reticulum membranes. It is suggested that the large amount of amorphous material liberated in the 5-day-old cells represents the unorganized enzymes and membranes.

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Résumé

L'expérience a été faite pour voir si les hépatocytes d'un poussin de 5 jours qui normalement ne produisent pas de réticulum endoplasmique lisse en culture, le feraient sous l'influence de la phénobarbitale. Les résultats ont indiqué que sous l'influence de la phénobarbitale, l'hépatocyte de 5 jours produit une petite quantité de réticulum endoplasmique lisse. Pourtant, une observation plus significative est que les hépatocytes de 5 jours en présence de la phénobarbitale produisent aussi une

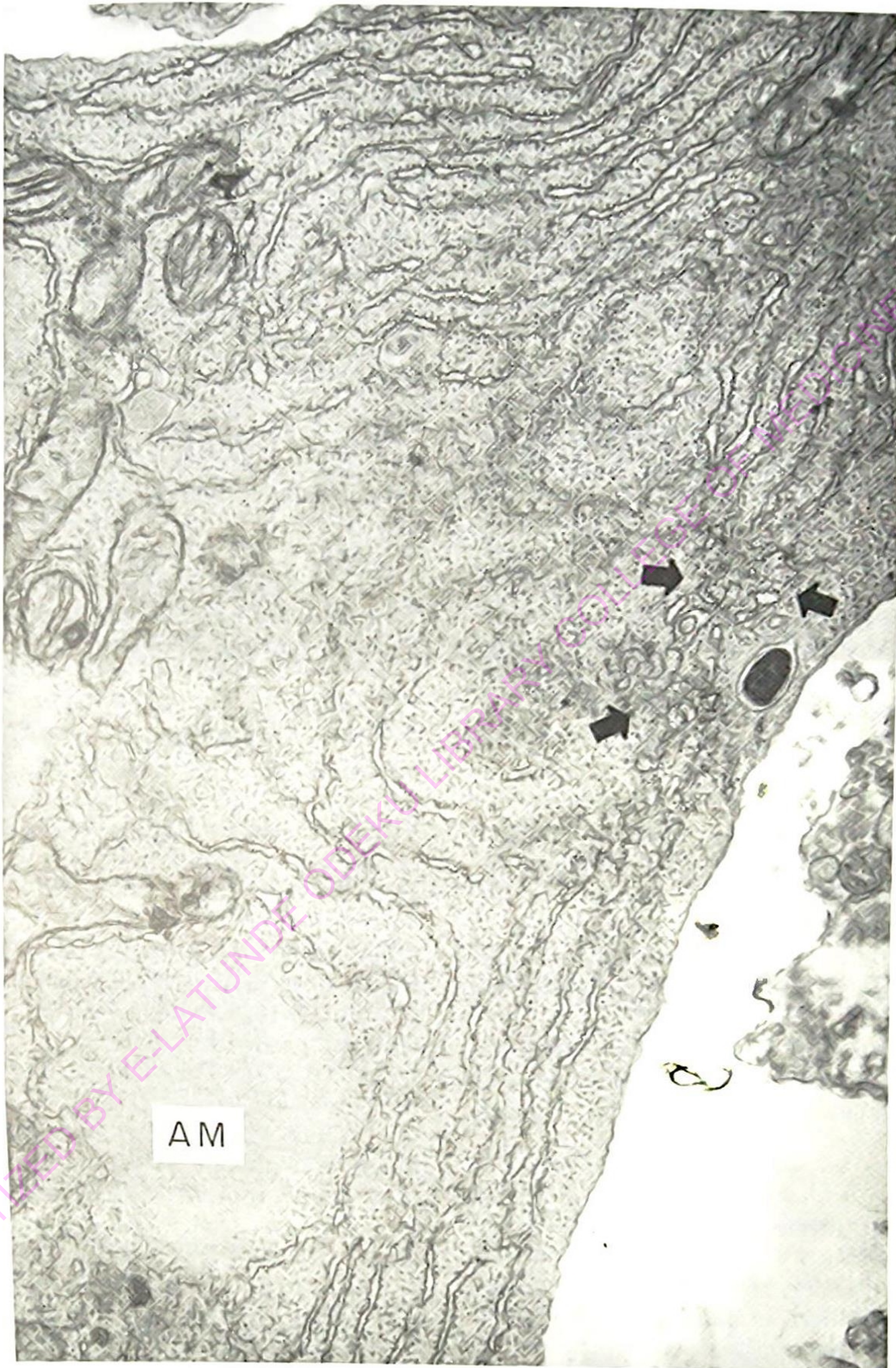

FIG. 1. Electron micrograph of a 5-day-old chick embryo liver cell after organ culture on rafts over Eagle's medium with 5.5 mM phenobarbital for 5 days. Observe the parallel membranes of the hypertrophied RER and the SER budding off the ends of the RER at the RER → SER conversion centre (arrows). Observe the translucent amorphous material within the cytoplasm (AM). (× 45 000).

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37.5°C 100% humidity and at atmospheric pressure for 2, 3 and 5 days. The first set of cultures were processed for electron microscopy at the end of 2 days, the second and third sets were processed at the end of 3 and 5 days respectively.

The tissues were fixed in modified buffered Karnowsky (Karnowsky, 1965) solution for 25 min at room temperature and pH 7.1. The fixative consists of 2.5% glutaraldehyde and 2% paraformaldehyde in a cacodylate buffer (0.1 M sodium cacodylate and 2 mM $CaCl_2$) at pH 7.1.

The tissues after rinsing in the cacodylate buffer, were post-fixed in cacodyalate buffered 1% OsO_4 at pH 7.1 and 0°C temperature for 20 min, and dehydrated in a graded concentration of ethanol solution to 100% at 0°C (1 × 2). The tissues were then placed in propylene oxide solution for 5 min and embedded in plastic mixture of Epon 312 and Araldite 502 (Banjo, 1978; Banjo & Nemeth, 1976; Mollenhauser, 1964). Thin sections of grey to pale gold interference colours were cut with a diamond knife, collected on copper grids, stained in a saturated solution of uranyl acetate in methanol and after rinsing in methanol were secondarily stained in 2% lead citrate solution as described before (Banjo, 1978; Banjo & Nemeth, 1976).

Observation was made with the J.E.O.L. 120 B electron microscope. The photographs were taken on Kodak E.M. film 4489 and the prints enlarged to a magnification of 20 000 and 45 000.

Estimation of the amount of the amorphous material produced was done as previously described (Banjo & Nemeth, 1976).

Results

The 5-day-old hepatocyte does not normally synthesize smooth endoplasmic reticulum in culture. But under the influence of phenobarbital, it produced a small amount of smooth endoplasmic reticulum which was proliferated from the ends of the rough endoplasmic reticulum at RER → SER conversion centres (Figs 1 and 6). However, the most striking manifestation of the 5-day-old hepatocytes was the formation of large quantities of amorphous material in the cytoplasm. These appeared as homogenous and finely granular areas in the cytoplasm and scattered within the amorphous material were varying shapes of membrane components (Figs 2 and 3).

The amorphous material appeared to be initially deposited inside the cisternal space of the rough endoplasmic reticulum which dilated into large vesicles. The dilated rough endoplasmic reticulum membrane apparently fragmented to liberate the amorphous material into the cytoplasm (Fig. 4).

The liver cells from the 11-day-old embryos also formed amorphous material but to a much lesser extent than the 5-day-old and none was formed until after 3 days of culture (Fig. 5).

In an earlier experiment, high dosages above 5.5 mmol resulted in the death of cells. Lower dosage did not appear to have any effect on the amount of amorphous substances produced.

The control cultures without phenobarbital did not form amorphous material (Fig. 7).

Discussion

Benzo and Della Haba (1972) have demonstrated that the 5-day-old chick embryo liver cell would produce smooth endoplasmic reticulum only if insulin hydrochloride or, better still, if zinc insulin was added to the culture. The amount of SER produced *in vitro* was small when compared to the amount produced by the same cell *in ovo* (Banjo & Nemeth, 1976). In this investigation it was observed that phenobarbital would also stimulate the production of smooth endoplasmic reticulum in the 5-day-old chick embryo liver cell in culture (Figs 1, 2 & 3). In this experiment there was an initial hypertrophy of the rough endoplasmic reticulum and a modest amount of smooth endoplasmic reticulum was formed at RER → SER conversion centres (Figs 1, 2 & 3).

The question could be asked, does the phenobarbital augment the effect of the insulin possibly present on the cell in the culture, in producing the smooth endoplasmic reticulum? Or is the smooth endoplasmic reticulum produced entirely due to the effect of the phenobarbital.

The condition of the experiment as explained below was such that there was no significant amount of insulin on the cell at

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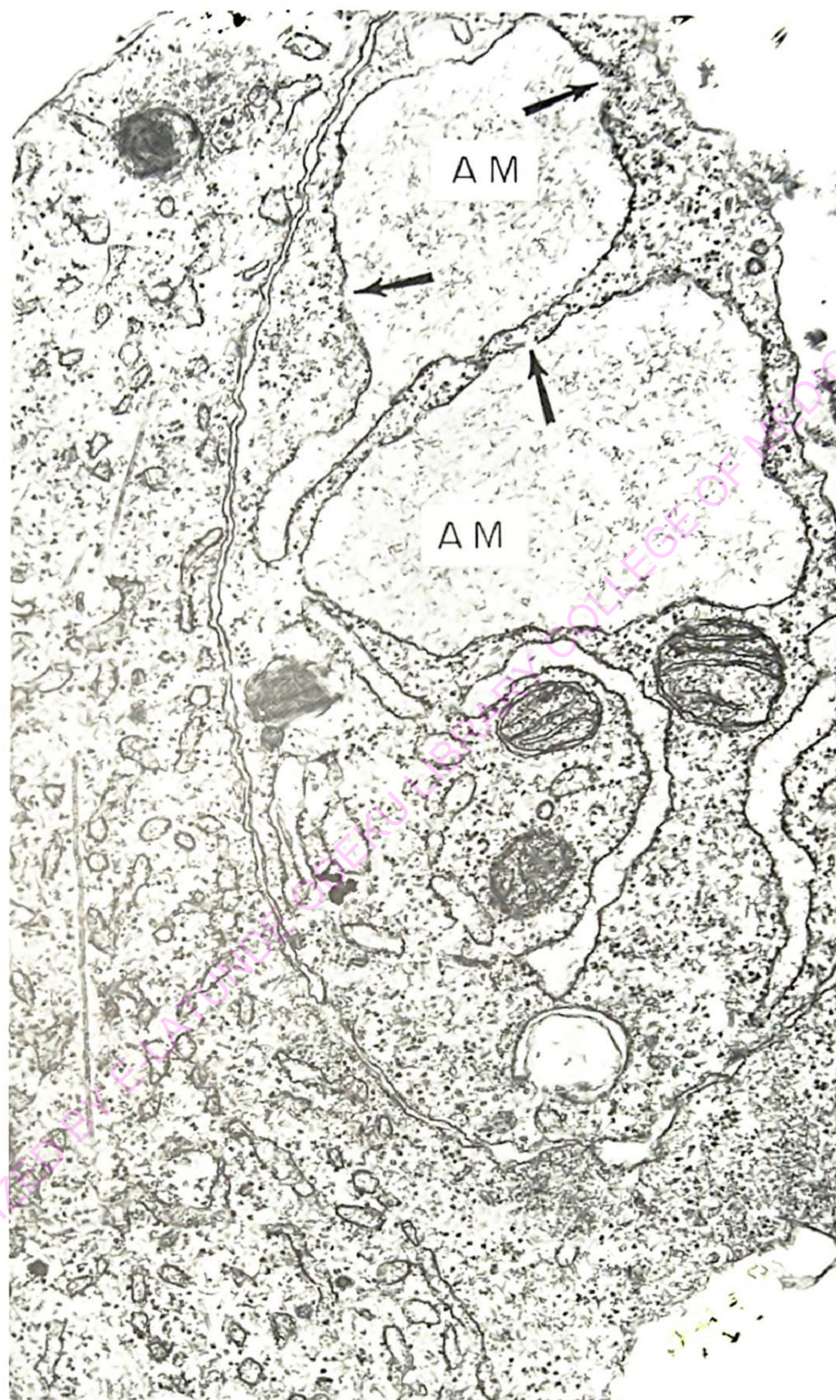

FIG. 4. Electron micrograph of a 5-day-old chick embryo liver cell after organ culture on rafts over Eagle's medium with 5.5 mM phenobarbital for 5 days. Note the dilated RER with amorphous material in their cavity. Note the points of break in the dilated membrane (arrows). AM, amorphous material. (× 45 000).

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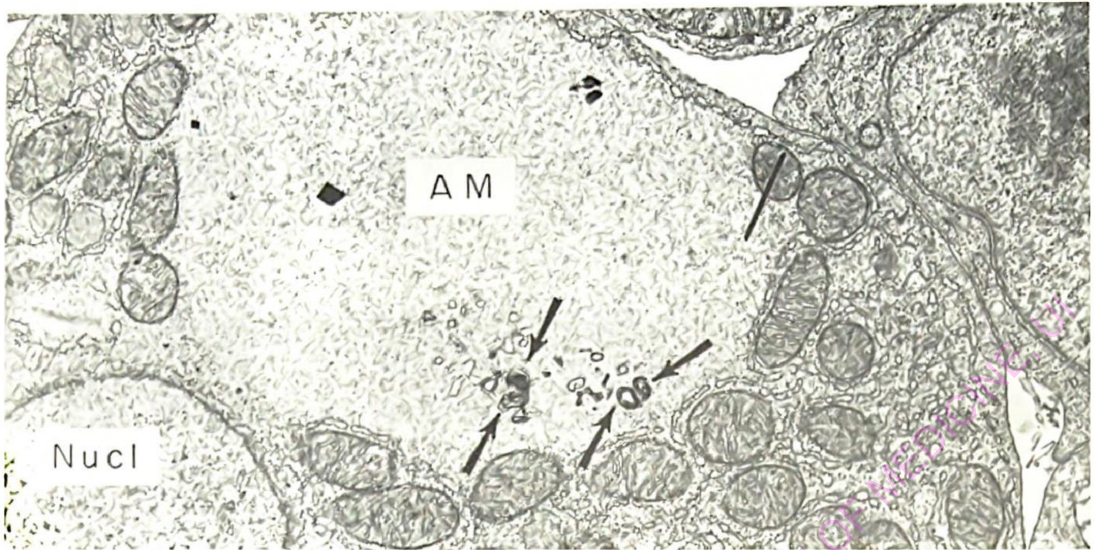

FIG. 2. Electron micrographs of 11-day-old chick embryo liver cells after organ culture on rafts over Eagle's medium with 5.5 mM phenobarbital for 5 days. Observe the translucent areas containing the material. Note that the nucleus is adjacent to the amorphous material. Note also the unorganized membranous material within the amorphous area. AM, amorphous material; Nucl, nucleus. (× 20 000).

FIG. 3. Electron micrograph of 11-day-old chick embryo liver cells after organ culture on rafts over Eagle's medium with 5.5 mM phenobarbital for 5 days. (cf. Fig. 2). (× 20 000).

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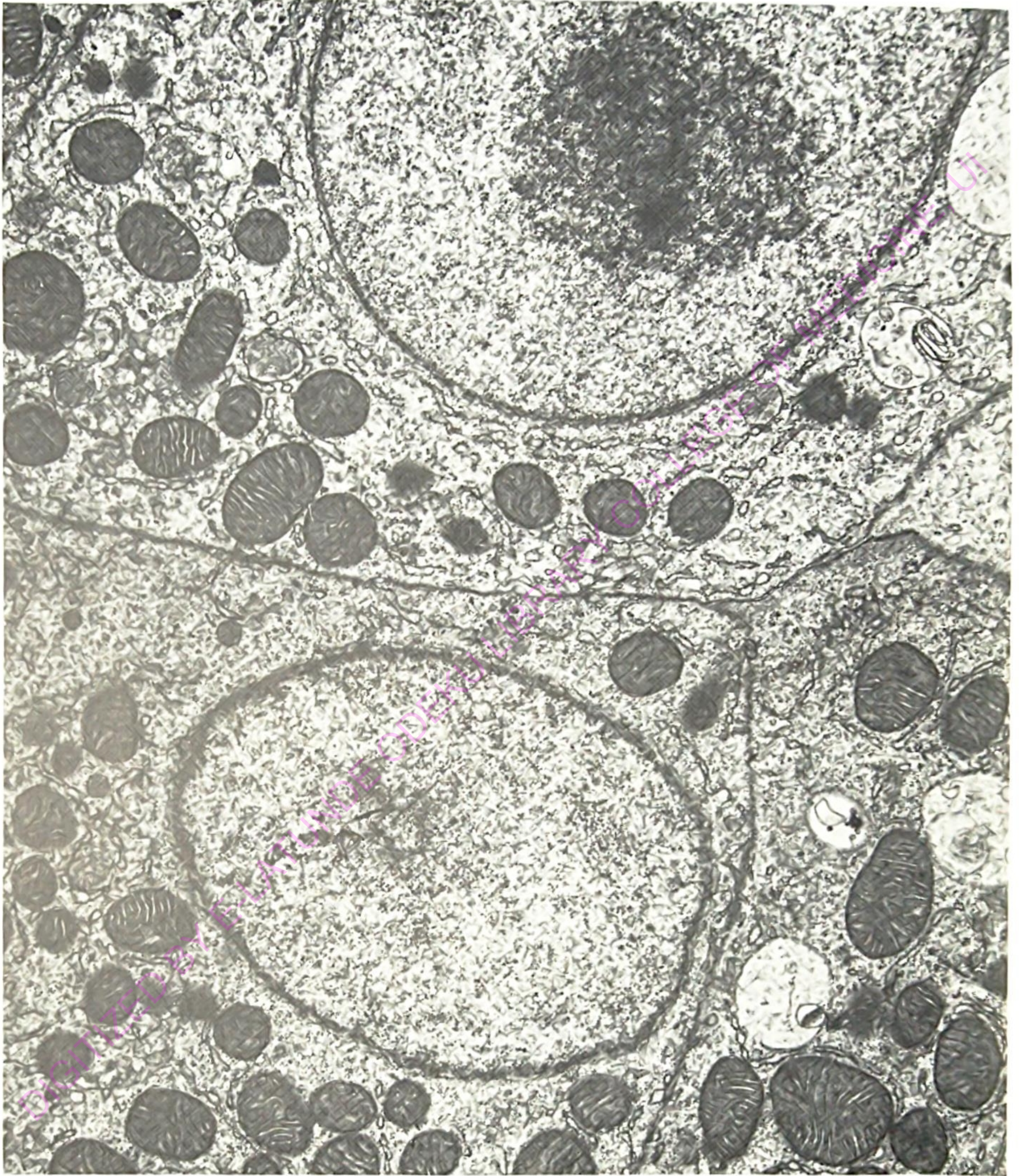

FIG. 7. Electron micrograph of a 5-day-old chick embryo liver after organ culture for 5 days without phenobarbital. Observe the moderate amount of RER and the complete absence of SER. Also note that there are no amorphous materials in the cytoplasm. (× 20 000).

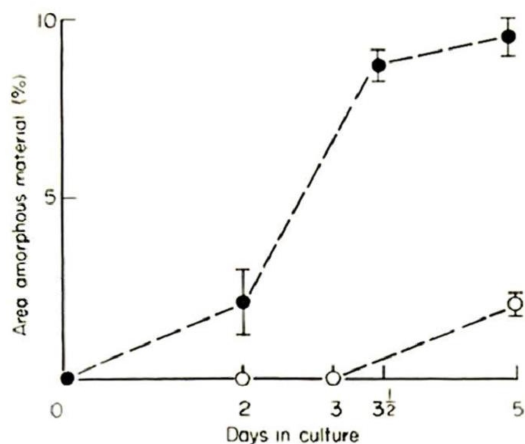

FIG. 5. The amount of amorphous material (μm^2) per unit of cytoplasm (μm^3) in 5- and 11-day-old chick embryo liver after organ culture on rafts over Eagle's medium with 5.5 mM phenobarbital. Each point represents the mean of the separate analysis of ten electron micrographs. The SEMs are indicated by vertical bars. Tissue from one embryo provided three and four micrographs.

the time of culture and 6 hr after culture there would be practically no insulin molecule left on the cell surface.

The liver specimens were exceptionally small, just a tiny fragment. They were washed for 3 min each in three changes of Simm's balance salt solution (Simms & Saunders, 1942) which would have removed any free insulin and also the insulin molecules which were detached from the cells' surfaces, since the attachment of insulin to the cell surface is reversible (Cautrecasas, 1972). More insulin molecules were lost from the cell into the culture medium in the dishes, and these were adsorbed onto the thin glass coating at the bottom of the culture dishes (Glass adsorbs insulin). The pH of the medium was 7.1 at which pH binding of insulin molecules to cell surface is unfavourable. Favourable binding occurs at pH 7.5 (Cautrecasas, 1974). When liver cell membranes were incubated at 37.0°C, 25% of the attached insulin were

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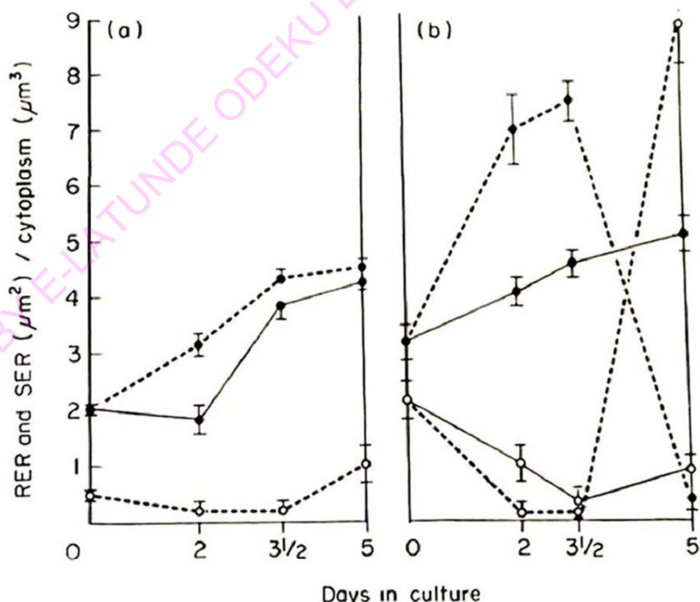

FIG. 6. RER (•) and SER (○) per unit of cytoplasm in (a) 5- and (b) 11-day-old chick embryo liver after organ culture on rafts over Eagle's medium with (- - - -) and without (——) 5.5 mM phenobarbital. Points represent the means of the separate analyses of ten electron micrographs. The SEMs are indicated by vertical bars. Tissue from one embryo provided three or four micrographs.

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degraded in one hour (Marinetti, Schlatz & Reilly, 1972). After all the above losses, one can safely assume that there was very little or no insulin molecules left on the cell surface just before culture. And after 6 hr of culture, one can safely assume that any insulin left on the cell surface would have been degraded.

In fact, when 0.2 ml serum from 17-day-old chick embryo containing 9.2 ± 0.6 μ/ml of insulin (Benzo & Green, 1974) were added to cultures of 5-day-old chick embryo liver cell containing phenobarbital and the cultures incubated for the same length of time as those containing phenobarbital alone, there was no increase in the smooth endoplasmic reticulum proliferated in the absence of added serum (Fig. 6). This suggests that the amount of insulin in the serum did not augment the effect of phenobarbital.

The mechanism involved in the formation of smooth endoplasmic reticulum in the presence of zinc insulin is not the same as in the presence of phenobarbital. Insulin enhances incorporation of amino acids into proteins whereas phenobarbital induces the production of new messenger RNAs (Essner & Novikoff, 1962; Gnosspelius & Orrenius, 1967) which act as template for the assembly of the molecules of the new drug-metabolizing enzymes (Ernster & Orrenius, 1965). Therefore, the smooth endoplasmic reticulum produced by both the 5-day-old and 11-day-old hepatocytes in the presence of insulin must be different in its enzyme content and structure from that produced in the presence of phenobarbital.

One can therefore postulate that the 5-day-old chick hepatocyte, although still very immature, is capable of producing smooth endoplasmic reticulum in culture in the presence of phenobarbital.

The 5-day-old hepatocytes formed a large amount of amorphous material (Fig. 5) which is probably hydrophobic as it occupied discreet areas and did not diffuse into the cytoplasm. The amorphous substance is also probably not simple lipid biochemically as it would have been dissolved away during processing for electron microscopy. It is not glycogen as it was not stained by lead citrate.

If as suggested that membrane bound enzymes, e.g. UDP-glucuronyl transferase (Banjo & Nemeth, 1976) and the other drug-hydroxylating enzymes (Ernster & Orrenius, 1965) which are synthesized as a result of phenobarbital stimulation are inseparably located on receptor surfaces of the endoplasmic reticulum, it could be that the large amount of drug-metabolizing enzymes produced could not find receptor surfaces for binding, especially in the 5-day-old hepatocytes with limited capacity to produce smooth endoplasmic reticulum; and therefore the enzymes become unorganized and/or re-discharged into the cisternal space of the rough endoplasmic reticulum which then dilated to liberate the unorganized enzymes and membranes into the cytoplasm (Figs 1 and 5). One may wonder whether the dilation of the RER and subsequent fragmentation of the RER membrane (Fig. 4) were artefact. But it is sufficient to say that none of these manifestations were seen in the control culture without phenobarbital (Fig. 7).

The 11-day-old hepatocytes on the other hand, formed very little amorphous substance (Fig. 5) and this may be due to its high capacity to make smooth endoplasmic reticulum.

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