The maturation of cortisone-treated embryonic duodenum in vitro. II. The striated border

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WITH FOUR PLATES

Regulation of the morphological and physiological maturation of embryonic duodenal mucosa by adrenocorticoid hormones has been demonstrated with a variety of techniques. Single injections of cortisone acetate (Moog & Richardson, 1955; Moog & Thomas, 1957); multiple daily administrations of cortisone (Moog & Ford, 1957; Moog, 1959); or in vitro maintenance of duodenal fragments with adrenal steroids (Moog & Nehari, 1954; Moog & Kirsch, 1955) all accelerate morphogenesis and the onset of alkaline phosphatase activity in this tissue.

The differentiation of the duodenal mucosa is not only responsive to hormonal application but is dependent on it. Hinni & Watterson (1963) have ablated pituitary glands from chick embryos, hence interrupting the hypophyseal-adrenal axis and arresting adrenal function. Following this procedure, morphogenesis is retarded and alkaline phosphatase activity in the epithelium diminished. Release from adrenocortical influence does not modify the course of differentiation, but does affect the rate of cellular maturation.

Implicit in the hormone-dependent quality of duodenal maturation is the existence of a delicate balance between the rate of tissue differentiation and the hormonal exposure. The present investigation has been conducted to examine this relationship between the concentration of hormone applied to explants in vitro and the responses induced in the epithelial striated border. This relationship has been studied in embryos representing a series of incubation ages to observe differential responsiveness of the tissue when explanted at various stages.

Materials and Methods

Duodenal loops from White Leghorn chicken embryos of stages 37–45 (Hamburger & Hamilton, 1951) were removed aseptically and minced into fragments 1–2 mm3 in size. These explants were maintained as organ cultures...
aboard a Millipore filter disc supported by a stainless steel grid (after Trowell, 1959). The nutritive medium was #199 (Morgan, Morton & Parker, 1950) supplemented with 10 per cent. calf serum. Cortisone acetate was added in the following concentrations: 0·01, 0·025, 0·05, 0·075, 0·1, 0·25, 0·5, and 1·0 \( \mu \text{g.} \)/ml.

All explants were fixed after 48 hr. of incubation at 37° C. They, as well as the unincubated control tissues, were fixed in 2 per cent. glutaraldehyde buffered with sodium cacodylate (280–320 mOs; pH 7·2–7·6) for 1 hr. at 4° C. and subsequently washed overnight in an isotonic solution of sucrose in cacodylate buffer (Gordon et al. 1963). For detection of alkaline phosphatase activity, the fixed tissues were incubated \textit{in toto} for 1 hr. at 37° C. in the presence of sodium \( \beta \)-glycerophosphate at pH 9·4. They then were reacted according to Gomori (1941) to produce a visible precipitate of cobalt sulphide. Sample tissues were incubated in media without substrate (glycerophosphate) to test for specificity of the reaction.

Quantification of enzyme activity was achieved by a procedure reported by Doyle (1950). After wet weights were obtained, controls and cultures were reacted for alkaline phosphatase by a modified Gomori procedure to yield a deposit of lead sulphide at sites of enzymatic activity. This deposit was extracted and reacted with \( \text{N,N-dimethyl-p-phenylenediamine sulphate} \) to synthesize methylene blue. After oxidation of this solution with ferric chloride, the content of methylene blue was determined by spectrophotometric transmission at a wave length of 670 m\( \mu \). Conversion of these optical readings to values per milligram of tissue yielded a series of quantitative estimates of enzyme activity (Table).

Tissues were prepared for histological observation by post-fixation in 1 per cent. osmium tetroxide in cacodylate buffer for 1 hr. at 4° C. Following alcoholic dehydration, they were embedded in Epon 812 (Luft, 1961) and sectioned on a Porter–Blum microtome. For light microscopic observation, 1·5 \( \mu \) thick sections were visualized by staining with 1 per cent. toluidine blue. Thin sections for electron microscopy were cut from the same blocks and stained with lead citrate (Venable & Coggeshall, 1965).

RESULTS

The localization of alkaline phosphatase activity

Inspection of thick sections of explants treated for alkaline phosphatase by the Gomori technique reveals no reaction product in 11-, 13- and 15-day control tissues. When cultivated without hormone, no change in activity is observed in these tissues. A positive reaction, however, is observed in 17-day controls. Also, the 19-day control is positive (Plate 2, Figs. E & F) and although this activity diminishes during cultivation without hormone, the reaction is not lost (Plate 2, Fig. G).

Addition of cortisone to the nutritive medium does not induce a positive...
<table>
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<th>Concentration of cortisone (µg./ml.)</th>
<th>13 Days</th>
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<th>17 Days</th>
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**Table**

Results of Doyle technique for quantification of alkaline phosphatase activity

Incubation Age of Embryonic Tissue
reaction for alkaline phosphatase in the 11-day culture (Plate 1, Fig. A), but does yield a reaction in the 13- and 15-day explants (Plate 1, Figs. B & C). The 17-day tissues continue to demonstrate positive activity after hormone treatment (Plate 1, Fig. D). Also, the 19-day tissue treated with hormone is positive for enzymatic activity (Plate 2, Figs. H & I).

No precipitate is found on those tissues incubated in medium lacking β-glycerophosphate but otherwise treated by the Gomori method. This is true even when controls or cultures of the same age are positive for alkaline phosphatase.

Where a positive reaction is obtained, the precipitated sulphide is located invariably on the luminal surface of the duodenal epithelium. This deposit is scattered irregularly over the surface of villi on isolated cells as well as small groups of cells. Specifically, the reaction product is localized on the microvillous membrane and along the apical cell membrane separating microvilli (Plate 3, Figs. J & K). Negatively-staining cells are found adjacent to positive ones, in which case the reaction product ends abruptly at the intercellular junction (Plate 3, Fig. J). Whenever the microvilli are positive, the apical cell membrane is also.

Quantification of alkaline phosphatase activity in embryonic duodenal tissue

Results obtained from application of the Doyle procedure for quantifying enzyme activity are presented in the Table. Spectrophotometric readings were corrected for variation in luminal surface area of duodenal explants by conversion to values per milligram of tissue. These values are significant within any one age group but are not comparable between age groups because of differences in thickness of the duodenal wall.

Alkaline phosphatase activity reaches a maximum at a definite hormonal concentration regardless of tissue age (Text-fig. 1). Increasing cortisone levels beyond this optimal concentration reduces the enzymatic response of the tissue. Moreover, the amount of cortisone necessary to induce maximal activity in duodenal explants decreases with increasing incubation age of the tissue. In other words, the older the tissue, the greater its sensitivity to the hormone (Text-fig. 2).

Morphological alterations in the striated border

The only dramatic change in morphology of the striated border occurs in the 19-day tissue following hormonal exposure. Compared to that of the untreated 19-day culture (Plate 2, Fig. G), the striated border of the hormone-treated epithelium is higher (Plate 2, Figs. H & I). Electron micrographs of this cortisone-treated epithelium reveal that the apparent increase in height of the border is due to the apposition of a layer of vesicles onto the microvilli (Plate 3, Fig. M; Plate 4). The thickness of the vesicular layer is 2-3 times the height of the microvilli (Plate 4, Fig. N). The combined height of the vesicular and micro-
PLATE 1

Hormone-treated cultures reacted for alkaline phosphatase by the Gomori technique.

Fig. A. 11-day duodenum cultivated with 0.25 μg./ml. cortisone. No visible enzymatic activity is observed. Toluidine blue, × 1400.

Fig. B. 13-day duodenum cultivated with 0.5 μg./ml. of cortisone. An irregular phosphatase reaction is localized on the lateral sides of villi. × 1400.

Fig. C. 15-day duodenum cultivated with 0.5 μg./ml. of cortisone. Alkaline phosphatase activity is shown on sides of villi. × 2000.

Fig. D. 17-day duodenum cultivated with 0.25 μg./ml. of cortisone. Individual cells and isolated groups of cells stain positively for phosphatase. × 1400.

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Plate 2

19-day controls and cultures stained for alkaline phosphatase.

Fig. E. Control tissue. Striated border (sb) stains positively. × 1400.

Fig. F. Control tissue. Note irregular enzymatic activity, which changes abruptly at intercellular junctions. × 1400.

Fig. G. 19-day duodenum cultivated 48 hr. with no hormone. × 1400.

Fig. H. 19-day duodenum cultivated 48 hr. with 0.01 μg./ml. of cortisone. The striated border is thicker and stains heavier than in (B) and more cells stain positively. × 1400.

Fig. I. 19-day duodenum cultivated 48 hr. with 0.5 μg./ml. of cortisone. The striated border is thicker than in G. × 2000.

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FIG. J. Electron micrograph of 19-day tissue cultivated for 48 hr. with no hormone and stained for alkaline phosphatase. Enzyme activity is localized on microvillous membrane and apical cell membrane between microvilli. Reaction ends at intercellular junction (arrow). × 24,100.

FIG. K. 19-day explant with 0.1 μg./ml. of cortisone and stained for alkaline phosphatase. Note phosphatase-positive vesicles and unstained hirsute coating (hc) of membranes.

FIG. L. 19-day control tissue showing absence of vesicular layer on microvilli. × 7000.

FIG. M. 19-day culture exposed to 0.1 μg./ml. of cortisone. Notice development of terminal web (tw) and presence of vesicular layer (ves) on luminal surface of microvilli (mv). × 6300.

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Plate 4

Electron micrographs of 19-day explants treated with 0.5 μg./ml. of cortisone for 48 hr.

Fig. N. Vesicular layer (ves) is 2–3 times thicker than the height of the microvilli. × 12,100.
Insert shows lack of internal structure in vesicles.

Fig. O. Apical extensions or distortions on microvilli might represent developing vesicles (arrows). × 17,800.

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Cortisone-treated embryonic duodenum

Alkaline phosphatase activity in 48-hour cultures of embryonic chick duodenum treated with cortisone

TEXT-FIG. 1. Alkaline phosphatase activity in the striated border of 48-hr. cultures of embryonic chick duodenum treated with cortisone. Induction of enzyme activity reaches a maximum at a definite hormonal concentration. Greatest response to the hormone is shown by 15- and 17-day tissues.

TEXT-FIG. 2. Cortisone inducing maximal alkaline phosphatase activity in embryonic chick duodenum maintained in vitro. Tissue sensitivity to the hormone increases as tissue age increases.
villous layers is comparable to the height of the border as viewed in light micrographs. The spheroid units comprising the vesicular layer are slightly smaller in diameter than the microvilli, are membrane-bound, and their external surfaces are covered with a hirsute coating as characterizes the surface of enteric microvilli (Ito, 1964). When stained for alkaline phosphatase, these extra-cellular spherules demonstrate positive enzymatic activity (Plate 3, Fig. K). Both the hirtellous coat and the phosphatase-positivity suggest that these vesicles are derived from the cell membrane.

Further indication of the origin of these vesicles is provided in micrographs showing apical distentions of the microvilli (Plate 4, Fig. O). These protrusions of the apical membrane of the microvillus suggest that the gemmules are pinched off from the tips of the microvilli. The surface filamentous coat retains these buds in contact with one another and with the cell surface. Hence, a definite layering of these vesicles on the luminal surface of the epithelium results. As hormone concentration is augmented, the striated border increases in height through a greater degree of vesiculation. During this process, the microvillus does not decrease noticeably in height, suggesting a concurrent expansion of the plasmalemma.

Although no internal structure is observed in the vesicles, there is a definite increase in filamentous material within the microvillus proper. This change resulting from hormone application is reflected in the development of the terminal web region of the apical cell cytoplasm (cf. Plate 3, Figs. L & M).

DISCUSSION

Our data demonstrate a positive effect of cortisone, under the cultivation conditions chosen, upon the maturation of the striated border of the embryonic duodenal epithelium. The normal rate of differentiation of these cells in vivo is approximated in vitro after hormonal administration. Untreated cultures fail to attain comparable stages of maturation either morphologically or physiologically.

The onset of functional activity, as evidenced by the accumulation of alkaline phosphatase in the tissue, is stimulated by cortisone. As originally demonstrated by Moog & Nehari (1954), this in vitro effect indicates a direct influence of the hormone on the duodenal tissue. This response reaches maximal levels with lesser concentrations of hormone as tissue age increases, provided the tissue is competent to respond. In the present study, enzymatic activity is not visualized in the 11-day tissue, but phosphatase activity is (1) induced in the 13- and 15-day cultures and (2) intensified in the 17- and 19-day tissues.

Maximal enzymatic activity is reached with a definite hormonal concentration such that submaximal levels are elicited by concentrations above or below this value. That is, the duodenal mucosa is increasingly responsive to cortisone up to a point where the induction of the phosphatase activity is optimal. This
intensification of enzyme activity is attributed both to an increase in the number of reactive cells and to an increase in reactivity per cell.

Once the optimal hormonal concentration is exceeded, maximal enzymatic activity is lost. Hence, cortisone has a repressive effect upon the enzymatic machinery of the cell when given in excess. Other studies indicate that over-exposure to steroids is generally toxic to tissues (Karnofsky et al. 1951; Evans, 1953; Grossfeld, 1959; Moscona & Karnofsky, 1960). Moreover, the degree of toxicity shown in vitro varies with the cell strain and the specific corticosteroid utilized (Gillette & Buchsbaum, 1953; Holden et al. 1953; Grossfeld & Ragan, 1954; Holden & Adams, 1957; Perlman et al. 1962).

Lasfargues & Di Fine (1951) have reported the diminution of alkaline phosphatase activity in 14-day tissue following administration of an adrenocortical extract, ‘eschatine’, but they did not vary the hormone concentration to reveal the extent of this effect. Moog & Nehari (1954) have attributed these negative results to the insensitivity of the tissue to hormone at that age as well as to cultivation conditions favoring the monolayering of cells. Our data, however, indicate that chick tissue is sensitive to corticoid influence as early as 13 days of incubation. Consequently, Lasfargues & Di Fine’s negative results might best be attributed to exposure to excess hormone levels, which the authors have expressed in ‘dog units’.

In spite of the evidence for positive effects of cortisone on the duodenal mucosa, other in vitro studies have suggested little or no dependence of the tissue upon cortisone. Hancox and Hyslop (1953) report that 15-day tissue, which exhibited positive phosphatase activity before cultivation, shows increased activity after 2–3 days in vitro without hormone. Hancox (1954) has reported that 12-day tissue, which is enzymatically negative at explantation, becomes positive after 4 days of cultivation. Moog & Nehari (1954) report that 16-day tissue responds similarly after 1–2 days in vitro. Such apparent indications of steroid independence can be explained by a latency in the appearance of the enzymatic activity. Adrenal function has been reported in the 12-day chick embryo (Dawson, 1953; Moog, 1959), so the tissue used in the above studies must have been exposed to the hormone. That this exposure, if sufficient, need not be continuous is quite probable.

In addition to influencing enzymatic activity on the apical cell membrane, cortisone induces morphological changes in the striated border. As hormone is applied to 19-day explants, the microvilli appear to elongate. This increase in length continues during exposure to hormone levels which coincidently repress enzyme activity. Dissociation between the morphological and physiological aspects of differentiation of duodenal epithelia has been reported previously by Kato (1959) and by Moog & Nehari (1954). These workers based their deduction on a change in enzymatic levels with no concurrent change in morphology, while our data indicate structural changes with no corresponding increase in phosphatase activity.
The height of the striated border normally increases during maturation. Microvilli elongate in vivo 6-fold between days 16 and 22, with the greatest change occurring at day 21. This increase in surface area of the microvilli parallels an increase in alkaline phosphatase activity along the apical cell surface (Overton & Shoup, 1964).

Exposure to cortisone in vitro stimulates an increase in apical plasmalemmal surface on the duodenal epithelium. This increase is complicated by vesiculation of the tips of the microvilli. Although the microvillous membrane is expanded, there is no significant increase in height of microvilli compared to 19-day controls.

Present attempts to explain vesiculation of microvilli are at best speculative. Other work with corticosteroids, however, might be linked with the results reported here. Fell & Thomas (1961) have demonstrated that hydrocortisone retards the resorptive effects of Vitamin A on skeletal tissue in vitro. The vitamin is thought to disrupt membrane integrity and to increase membrane permeability (Lucy, 1964). These effects are exerted on both the plasmalemma (Dingle & Lucy, 1962; Dingle et al. 1962; Glauert et al. 1963) and intracellular membranes (Dingle, 1961; Fitton Jackson & Fell, 1963; Lucy et al. 1963). Hydrocortisone is thought to counteract the influence of Vitamin A by stabilizing the membrane systems of the cell.

Cortisone, likewise, might increase the integrity or stability of the apical cell membrane and in so doing might inhibit increases in the surface area of this portion of the cell membrane. If so, the hormone would be stimulating precocious membrane-bound phosphatase activity, yet concomitantly restricting membrane expansion which is linked developmentally to and which varies directly with enzymatic activity.

Vesiculation of the cell membrane, under the influence of cortisone, might represent the cell's attempt to maintain equilibrium between the physiological and morphological phases of differentiation. The small portions of the membrane and cytoplasm released as vesicles would no longer be identified as part of the cell. Consequently, when the duodenal epithelium is exposed to concentrations of cortisone greater than that yielding maximal enzyme activity, no disproportionate increase in apical surface area results.

**SUMMARY**

1. The effects of cortisone on the striated border of the embryonic chick duodenal epithelium during in vitro maintenance are described.

2. The application of cortisone induces phosphatase activity in 13- and 15-day tissue and intensifies this activity in 17- and 19-day explants.

3. The phosphatase activity obtained varies in quantity depending on the age of the tissues and the concentration of hormone. The sensitivity of the tissue to hormone increases with age; that is, a lower cortisone concentration is needed to elicit a maximal enzymatic response in older tissues. As hormone concentration
Cortisone-treated embryonic duodenum

is increased for all explants from 13 to 19 days, phosphatase activity increases to a maximum and thereafter decreases.

4. Cortisone induces a morphological change in the striated border of 19-day tissue. The apical ends of microvilli are pinched off to form numerous spheroid vesicles. A possible explanation for this phenomenon is discussed.

RESUME

La maturation in vitro du duodenum embryonnaire traité par la cortisone

II. La bordure striée


2. L’application de cortisone induit l’activité phosphatasique dans le tissu de 13 et de 15 jours et intensifie cette activité dans les explants de 17 et de 19 jours.

3. L’activité phosphatasique obtenue varie quantitativement selon l’âge du tissu et la concentration en hormone. La sensibilité du tissu à l’hormone s’accroît avec l’âge; c’est-à-dire que plus les tissus sont âgés plus la concentration de cortisone nécessaire pour produire la réponse enzymatique maximum est basse. Lorsqu’on augmente la concentration d’hormone pour tous les explants de 13 à 19 jours, l’activité phosphatasique augmente jusqu’à un maximum puis décroît.

4. La cortisone induit un changement morphologique de la bordure striée dans le tissu provenant d’embryons de 19 jours. Les terminaisons apicales de microvillosités se fragmentent pour former de nombreuses vésicules plus ou moins sphériques. Une explication possible de ce phénomène est discutée.

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REFERENCES


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