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THE EFFECTS OF REGIONAL HOMOGRAFTS OF THE PARS DISTALIS
ON THE DEVELOPMENT OF THE DUODENUM OF CHICK EMBRYOS

by

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A thesis submitted to the Faculty of
Graduate Studies in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

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ABSTRACT

The pars distalis or anterior pituitary gland of the chick embryo has two distinct regions, the cephalic and caudal. Histochemical and physiological evidence indicates that each of these regions contains certain hormone activities distinct from the other.

Various workers have reported that the adreno-cortico-pars distalis and the thyro-pars distalis axes are essential for the normal pattern of duodenal maturation in the chick embryo during the final stages of incubation. The involvement of additional pars distalis hormones has also been suggested.

The role of the pars distalis in duodenal development was further explored with a regional grafting technique. Embryos hypophysectomized at 33 hours of incubation were subsequently given a graft of a specific region of the pars distalis and autopsied at one-day intervals from 17.5 through 20.5 days of incubation.

Histology of grafts and thyroid glands and fixed weights of thyroid glands indicated that most grafts were successfully established and functioning.

Hypophysectomy resulted in greatly increased mortality and a characteristic syndrome of defective growth and external features. Cephalic and caudal region grafts tended to reduce mortality but only cephalic grafts effected repair of the defective exter-

nal features. Retarded body growth (assessed by fresh body weights and third toe lengths) of hypophysectomized embryos was considerably improved by cephalic region grafts but not by caudal region grafts.

Hypophysectomy caused severe retardation of duodenal growth and differentiation assessed in terms of fresh weight, total protein content and specific activity of alkaline phosphatase. Mucosal cell morphogenesis was arrested at a level equivalent to 17.5 days of normal development. Cephalic region grafts repaired the defects in duodenal ontogeny of hypophysectomized embryos. Two cephalic region grafts or entire pars distalis grafts achieved no greater repair than a single cephalic region graft indicating that cephalic region grafts secreted sufficient levels of hormone to elicit the maximum response. Alternatively, a possible feedback system not mediated by the hypothalamus may have prevented double grafts from secreting excess levels of hormone.

Single or double grafts of caudal region did not appear to participate in duodenal development. The ineffectiveness of single caudal region grafts evidently was not due to insufficient levels of hormone and the different effects of the cephalic and caudal regions reflects a qualitative difference in the hormone activities of each.

Grafts of cephalic and caudal regions in combination were not as effective in repair of duodenal defects as cephalic region grafts alone indicating a possible inhibitory interaction between regions. This problem requires further study, however.

Dietary albumen tended to augment duodenal growth and differentiation.

Probably the cephalic graft normalizing effects are due to the ACTH and TSH support of normal titers of adrenocortical and thyroid hormones which may directly participate in duodenal development. STH is probably not an essential addition and it alone probably can not cause normal development. Prolactin may mimic the development or growth-promoting effects of STH but the evidence as yet is not compelling.

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INTRODUCTION

The morphology and development of the vertebrate small intestine (in particular, the duodenum) has been the subject of numerous investigations. Toner (1968) has made a comprehensive survey of the literature in this area.

In the course of duodenal development a variety of striking changes occur. These features have been particularly well explored in the duodenum of the chick embryo (Hilton, 1902; Moog, 1950; Moog and Richardson, 1955; Moog and Thomas, 1957; Coulombre and Coulombre, 1958; Moog, 1961; Hinni and Watterson, 1963; Bellware and Betz, 1970) and are generally recognized as landmarks in the normal pattern of differentiation.

The Maturation of the Duodenum

During the latter part of the first week of incubation development of the chick duodenum mainly involves the proliferation of tissues, especially of the epithelial mucosa, by extensive mitotic activity (Coulombre and Coulombre, 1958). Between 8 and 14 days of development the mucosa becomes thrown into folds, (the previllous ridges) (Hilton, 1902; Coulombre and Coulombre, 1958). Furthermore the ridges appear in a geometric progression, new ridges arising between

pre-existing ones, e.g. at 8-9 days, 2 folds or ridges; at 9-10 days, 4; at 10-11 days, 8; etc. (Coulombre and Coulombre, 1958; Hinni and Watterson, 1963). The fifth and final rank of previllous ridges (extending the total to approximately 32) is completed shortly after the 16th day of development. Between 11 and 13 days of incubation, the previllous ridges already present become compressed into a zigzag pattern (Hilton, 1902; Coulombre and Coulombre, 1958). Indentations subsequently appear in the angles of the zigzags thereby delimiting the villi. Generally villi begin to appear at 16 days with extensive elongation of the villi occurring between 18 days and hatching (Coulombre and Coulombre, 1958; Hinni and Watterson, 1963; Bellware and Betz, 1970). According to Clarke (1967) growth of the villi beyond 19 days is confined to an increase in size without addition of new villi.

Mitotic figures continue to occur throughout the mucosa until 15 days of development thereafter becoming progressively confined to the bases of the villi and ultimately to the crypts of Lieberkühn at hatching (Hinni and Watterson, 1963; Overton and Shoup, 1964). This confinement is characteristic of the adult intestinal mucosa in many mammals as well as in the chicken and mitotic activity in the crypts is an essential part of the mucosal cell renewal system (Hinni and Watterson, 1963; Imondi and Bird, 1966; Toner, 1968).

This system involves proliferation of new cells (from the crypts) which migrate up the sides of the villi maturing en route, to replace the cells which are shed into the lumen from extrusion zones at the villus tip (Toner, 1968). Such extrusion zones are present in the chick duodenum a day or so after hatching (Moog, 1950; Hinni and Watterson, 1963).

Von Pap in 1933 (fide Romanoff, 1960) described the degeneration of mesenchyme cells in the stroma of the ridges and noted a concomitant appearance of erthrocyte aggregations which often dilated the tips of the previllous ridges. He suggested these might be the first true villi. Moog (1961) observed swelling of the tips of villi at 16 days. Concomitantly the stroma becomes less dense. She ascribed this to an increase in vascularization. The erthrocyte aggregations as well as dilated villus tips disappear by 20 days (Bellware and Betz, 1970) and it is not known whether or not their presence represents a temporary hemopoietic function for the stroma.

The epithelium remains syncytial until 9 days of development and becomes cellular between 10 and 16 days (Argeseanu and May, 1938; Moog, 1950). During this period many cytoplasmic changes occur and various organelles assume their definitive adult positions (Argeseanu and May, 1938).

The first evidence of goblet cell primordia appears at the base of the previllous ridges between day 14 and 15 of incubation (Hinni and Watterson, 1963). These cells migrate from their basal positions and progressively mature so that by 19 to 20 days goblet cells occur throughout the mucosa including the apices of the villi (Hinni and Watterson, 1963; Bellware and Betz, 1970).

From 13 to 17 days, the absorbing cells remain cuboidal to low columnar with round nuclei, but thereafter become increasingly elongated (especially during the week after hatching) with ovoid nuclei (Moog and Richardson, 1955; Kato, 1959; Moog, 1961; Hinni and Watterson, 1963; Bellware and Betz, 1970).

Cytoplasmic basophilia removable by treatment with ribonuclease (Hinni and Watterson, 1963) or with cold perchloric acid (Moog, 1961; see also Richardson et al., 1955) is present as early as 13 days in the epithelial cells. It increases in intensity and becomes confined to the apical cytoplasm between 15 and 19 days of incubation. At 19 days a "wedge" of cytoplasm proximal to the nucleus exhibits a moderate intensity of basophilia which persists while apical basophilia almost disappears by day 20 (Moog and Richardson, 1955; Kato, 1959; Moog, 1961; Hinni and Watterson, 1963). The basophilia is presumably due to RNA, and the above changes would indicate shifting

loci of protein synthesis (Hinni and Watterson, 1963). According to Toner (1968) the basophilia, due to 'free' ribosomes, tends to disappear as ribosomes become membrane-bound during maturation (Webster and Harrison, 1969).

Glycogen is also present throughout the epithelial cell cytoplasm on the 13th day (Hinni and Watterson, 1963) and subsequently tends to accumulate in increasing amounts distal to the nucleus reaching a peak at 18 days and then declining until only traces remain at 20 days of incubation (Moog and Richardson, 1955; Moog and Thomas, 1957; Kato, 1959; Moog, 1961; Hinni and Watterson, 1963; Bellware and Betz, 1970).

The striated border begins to develop between 10 and 16 days (Argeseanu and May, 1938) and a definite although scattered microvillous pattern is evident by day 11 (Overton and Shoup, 1964). Intensive differentiation of the border ensues (between 16 and 22 days) (Overton and Shoup, 1964; Bellware and Betz, 1970). Before the border becomes apparent at 16 days, there is a faint Alcian blue positive material (indicating the presence of an acid mucopolysaccharide) present on the luminal surface of the epithelium (Hinni and Watterson, 1963) until day 19 but by day 20 it disappears. It may be identical to the "fuzzy" surface coat seen in the electron microscopic studies of the striated border (Fawcett, 1965; Toner, 1968; Ito,

1969).

Approximately one day after the appearance of the Alcian blue positive material (about day 15), the luminal surface of the epithelial cells exhibits a "patchy" PAS-positive reaction which is insensitive to amylase treatment. This material, a neutral or possibly basic mucopolysaccharide becomes progressively more concentrated as the striated border matures (Moog and Wenger, 1952; Moog and Richardson, 1955; Kato, 1959; Moog, 1961; Hinni and Watterson, 1963; Bellware and Betz, 1970).

Certain enzymes increase in activity concurrently with the maturation of the striated border, e.g. alkaline phosphatase(s) (Moog, 1950; Moog and Richardson, 1955), non-specific esterase(s) (Richardson *et al.*, 1955) invertase (Hijmans and McCarty, 1966) and leucine aminopeptidase and β -galactosidase (Baxter-Grillo, 1970). The duodenum has been recognized as one of the richest sources of alkaline phosphatase(s) (first demonstrated histochemically in this organ by Gomori, 1941). This enzyme system exhibits a dramatic 100-fold increase in activity between 17 and 21 days in the chick duodenum (Moog, 1950; Moog and Richardson, 1955; Kato, 1959; Moog, 1961). This has been confirmed by others in the chick (Hinni and Watterson, 1963; Bellware and Betz, 1970), and a similar developmental pattern for phosphatase activity has been

shown in the mouse (Moog, 1951) and in the guinea pig (Moog and Ortiz, 1960). Alkaline phosphatase activity has been closely correlated with several developmental features in the duodenum. The upsurge in activity during the last few days of incubation in the chick parallels a similar increase in the PAS-positive border reaction (Moog and Wenger, 1952). Moreover, there is good evidence that this enzyme contains a specific carbohydrate moiety (Moog and Wenger, 1952; Saini and Done, 1970). Various aspects of the structural changes during maturation of the absorbing cells have also been shown to be intimately associated with changes in alkaline phosphatase activity (Moog and Richardson, 1955; Overton and Shoup, 1964; Webster and Harrison, 1969). Although its relationship to duodenal differentiation is not understood, alkaline phosphatase activity has been extensively employed as an indicator of duodenal differentiation and several observations indicate the enzyme may have a role in transport processes. Alkaline phosphatase activity has been associated in electron microscopic histochemical studies with structures involved in fat and glucose absorption (Hugon and Borgers, 1966). The activity of the enzyme is localized principally in the region of the striated border (Gomori, 1941; Goldfischer *et al.*, 1964; Moog and Richardson, 1955; Kato, 1959; Holt and Miller, 1962;

Hinni and Watterson, 1963; Hugon and Borgers, 1969; Bellware and Betz, 1970) and substantial evidence has accumulated indicating that alkaline phosphatase is intimately associated with, if not an integral part of the microvillar membrane (Eichholz, 1969). Moreover, in a concurrent investigation in this laboratory (D. Mallon, unpublished observations), preliminary results have shown (in the chick embryo duodenum) that the development of the ability to absorb glucose against a concentration gradient parallels the increase in alkaline phosphatase activity measured in the same duodena. Indeed, it should be noted that the period during which intense maturational changes (including increases in alkaline phosphatase activity) are occurring in the intestinal epithelium coincides generally with the time at which active transport systems are developing (Bogner and Haines, 1964; Deren et al., 1965; Bogner et al., 1966; Holdsworth and Wilson, 1967; Taube and Berlin, 1970).

The Role of the Endocrine System in Duodenal Maturation

An increase in alkaline phosphatase activity (similar to that in the chick embryo duodenum) occurs just prior to weaning in the duodenum of the nursing mouse and this upsurge takes place precociously if the animals are stressed (Moog, 1951). Furthermore, exogenous adrenocorticoids or ACTH also elicit this

precocity and conversely, adrenalectomy inhibits alkaline phosphatase accumulation in the mouse duodenum (Moog, 1953). Likewise precocious accumulation of alkaline phosphatase activity in the duodenum of the chick embryo occurs in response to administration of adrenocorticoids (Moog, 1952; Moog and Richardson, 1955). A variety of other parameters of duodenal differentiation e.g. epithelial and villous morphogenesis, were similarly affected by these hormones suggesting that the adrenocortical hormones stimulate some fundamental process(es). Apparently this involves the entire sequence of developmental events in the duodenum (Moog and Richardson, 1955). In support of this concept the authors noted that (with the exception of glycogen accumulation which exceeded the normal maximum) (Moog and Thomas, 1957), for the parameters associated with this precocious inductive response, maximum stimulation did not surpass the upper limit of the normal developmental pattern at 19 days. Although the developmental pattern was premature, its integrity was apparently maintained. Moog and Richardson (1955) concluded that the pituitary-adrenal axis probably plays an important role in normal duodenal differentiation.

Subsequently, Moog and Ford (1957) reported elevated duodenal alkaline phosphatase levels as early as the fifteenth day of incubation in chick embryos which received ACTH intravenously. Moreover, in vitro

studies have shown that adrenocorticoids are capable of maintaining normal or elevated levels of differentiation in cultured explants of chick duodenum (Moog and Nehari, 1954; Moog and Kirsch, 1955; Hayes, 1965a; 1965b; Hijmans and McCarty, 1966). Much of the work relating to function of the embryonic adrenal cortex has been reviewed by Moog (1959a) and the development of a functional pituitary-adrenal axis has been reviewed by Betz (1971).

Thiourea injected into chick embryos retarded duodenal growth and morphogenesis (Moog, 1961; Stocum, 1966). The duodenum of thiourea-treated embryos appeared insensitive to the inductive effect of corticoids (Moog, 1961). These effects were alleviated by the administration of L-thyroxine, but this hormone failed to stimulate measurable precocity of duodenal development in normal embryos. Moog (1961) suggested that thyroxine is necessary for maintenance of a normal rate of differentiation and it appears to play a permissive role by inducing the sensitivity of the duodenal epithelium to corticoids.

The importance of the pituitary gland to normal duodenal development was indicated by Hinni and Watterson (1963). They partially decapitated chick embryos surgically, an operation which has effects equivalent to adenohipophysectomy (Betz, 1967; 1971).

In the absence of the pituitary, duodenal growth and differentiation did not exceed the normal level for 17 days of incubation (Hinni and Watterson, 1963).

In an attempt to restore the defective duodenal morphogenesis, these workers administered thyroxine and/or an adrenocorticoid to the hypophysectomized embryos but observed no apparent correction. Furthermore, Stocum (1966) reported an inhibition of duodenal growth by cortisone acetate in normal chick embryos but the results were not clear cut and the effects of the corticoid on morphogenesis of the villi were difficult to interpret. One possible conclusion was that the defects in duodenal ontogeny were not due solely to a lack of normal titers of thyroid and adrenocortical hormones (in the absence of TSH and ACTH stimulation of the thyroids and adrenal cortices) but that one or more additional hormones may be essential for restoration of normal duodenal development (Hinni and Watterson, 1963). STH was considered a likely candidate (see Hinni and Watterson, 1963) since it has been shown to have a mitogenic effect on the duodenal mucosa of rats (Leblond and Carriere, 1955). Specifically, mitoses in the mucosa of rats were arrested in hypophysectomized and/or thyroidectomized individuals but could be restored by thyroxine only in the presence of a source of growth hormone (Leblond and Carriere, 1955). The authors suggested that thyroxine may mediate the secretion of

STH by the pituitary (Leblond and Carriere, 1955; Carriere, 1966).

Recently, Bellware successfully repaired the defects in duodenal ontogeny in hypophysectomized embryos with chorioallantoic pars distalis homografts (Bellware and Betz, 1970). Alkaline phosphatase activities as well as most histological parameters were restored to normal levels in operated embryos by the time of hatching. These results are consistent with the hypothesis that STH may be essential for normal duodenal development. Also Struthers and Betz (unpublished data) reported increased levels of alkaline phosphatase in explants of chick embryo duodenum as compared with controls from both normal and hypophysectomized embryos when porcine STH was added to the culture medium. On the other hand, in pilot experiments porcine STH failed to alter in vivo duodenal phosphatase activities in both normal and hypophysectomized embryos. However, thyroxine and hydrocortisone each induced precocious alkaline phosphatase levels in the same experimental series (Betz and Mallon, 1970). In addition thyroxine and hydrocortisone together apparently had additive effects.

The above evidence indicates that the adrenocortico-pars distalis and thyro-pars distalis axes are important for normal duodenal development in chick embryos. One or more additional pars distalis hormones,

e.g. STH and/or prolactin may be important.

The Regions of the Pars Distalis

The pars distalis of the chick embryo is bilobed by the end of the first week of development, (Rahn, 1938). Moreover, the two regions (cephalic and caudal) have different staining characteristics (Rahn, 1939) and this evidently is a general avian feature (Rahn and Painter, 1941, see also Wingstrand, 1963). Several authors have confirmed the distinction of cephalic and caudal regions in the chick pars distalis on morphological and histological bases, but the specific details regarding the number of unique cell types are difficult to correlate (Payne, 1944; Wilson, 1952; Aronson, 1952; Mikami, 1954; 1958; Grignon, 1956; Wingstrand, 1963; Tixier-Vidal, 1963). There have been many attempts to relate specific cell types to specific hormonal activities by correlating changes in pars distalis cytology with natural or experimental alterations in endocrine physiology. For example, a certain type of cephalic acidophil, the 'broody cell' or 'A₂ cell' shows a marked hypertrophy during broodiness and is thought to secrete prolactin (Payne, 1944; Wingstrand, 1963). 'Thyroidectomy cells' or 'T cells' (those which hypertrophy in response to thyroidectomy) are basophils which occur near the boundary between the two regions (Payne, 1944; Wilson, 1952). Although

there is some doubt, (Phillips, 1962; Tixier-Vidal, 1963) these cells (probably the source of TSH activity) are apparently confined to the cephalic region in the chick (Mikami, 1954; 1958; Aronson, 1952; Grignon, 1956). 'Adrenalectomy cells' are also confined to the cephalic region and are presumably the source of ACTH (Grignon, 1956; Mikami, 1958; Tixier-Vidal, 1963). 'Castration cells' are basophils occurring in both regions and there has been some confusion of identities between the cephalic 'castration cells' and 'thyroidectomy cells' (Aronson, 1952) but Mikami (1958) recognizes a distinction between the two. The castration cells probably represent two different types of gonadotrophs (Mikami, 1958) and at least in the mallard the cephalic gonadotrophs are believed to secrete FSH; the caudal gonadotrophs, LH (Tixier-Vidal, 1963).

Bioassay of cephalic and caudal region hormone activities support the cephalic localization of TSH (Mikami 1958; Brasch and Betz, 1971); ACTH (Mikami, 1958; Brasch and Betz, 1971; see also Tixier-Vidal, 1963); and FSH (Brasch and Betz, 1971). Prolactin, however, appears to be produced in both regions (Nakajo and Tanaka, 1956). LH activity is caudally localized (Brasch and Betz, 1971) as is STH (Enemar, 1967). To summarize, there is substantial evidence that the cephalic region, exclusively, secretes TSH, ACTH, and FSH, and the caudal region, exclusively,

secretes LH and STH. The origin of prolactin is less certain (Brasch and Betz, 1971).

Thus the preceding evidence indicates that the chick pars distalis has cephalic and caudal regions which are anatomically distinct. This distinction is also reflected by histological characteristics and the different hormonal activities of each region. In view of the importance of the pars distalis in maintaining normal rates of duodenal differentiation (Bellware and Betz, 1970), the technique of regional grafting provides a more critical analysis of the specific pars distalis hormones involved in duodenal development. Thus, the effects of regional pars distalis grafts on duodenal differentiation in the chick embryo represent the subject of this thesis.

MATERIALS AND METHODS

A. Biological

General

White Leghorn eggs (Hyline strain 934F) from Neuhauser Hatcheries, St. Felix de Valois, Quebec, were used in this study. The eggs were incubated, without rotation, on foam rubber nests at $38^{\circ} \pm 0.5^{\circ} \text{ C}$ and $60\% \pm 5\%$ relative humidity. Variables introduced by prolonged preincubation storage (Arora and Kosin, 1966) were avoided by starting incubation within one week of laying.

Five major groups were in the experimental design: (1) normal embryos, (2) hypophysectomized embryos, (3-5) hypophysectomized embryos which received one homograft of either cephalic or middle or caudal regions of the pars distalis, respectively. Groups (1) and (2) were killed at one-day intervals from 15.5 through 20.5 days of incubation and groups (3-5) were killed daily from 17.5 through 20.5 days of incubation.

In an effort to test dosage effects, possible interaction between regions and to investigate the variables of the grafting technique, six ancillary groups were included in the study: (6) hypophysectomized embryos which received a homograft of the entire pars distalis, (7-9) hypophysectomized embryos which

received double homografts of two cephalic or two middle or two caudal regions of the pars distalis, respectively, (10-11) hypophysectomized embryos which received homografts of either cephalic and caudal regions of the pars distalis, or homografts of cephalic, middle and caudal regions. Groups (6-11) were killed at 20.5 days of incubation. The experimental design is summarized in Table 1.

Hypophysectomy

All manipulations of the embryos were performed through a circular window in the shell. First, the shell at the blunt end of each egg was punctured with a dissecting needle. Each egg was then placed on a specially designed mount on a small drill press with a cone-shaped cutting tool impregnated with diamond dust. A shallow circular cut 9 mm in diameter was made in each shell so as to leave the underlying shell membranes intact. The shell fragment was then removed and the shell membranes were pierced permitting the contents of the egg to settle away from the window. The membranes were discarded and the window was sealed with water-proof tape.

Embryos were hypophysectomized by surgical partial decapitation (Betz, 1965) at 33 to 38 hours of incubation. The embryos are at stage 10 or 11 and have 10-13 pairs of somites (Hamburger and Hamilton, 1951).

TABLE 1 EXPERIMENTAL DESIGN

Group	Type of Graft	Days of Incubation
1	Normal - no graft	15.5-20.5
2	Hypophysectomized - no graft	15.5-20.5
3	Hypophysectomized + 1 cephalic region	17.5-20.5
4	Hypophysectomized + 1 middle region	17.5-20.5
5	Hypophysectomized + 1 caudal region	17.5-20.5
6	Hypophysectomized + 1 entire pars distalis	20.5
7	Hypophysectomized + 2 cephalic regions	20.5
8	Hypophysectomized + 2 middle regions	20.5
9	Hypophysectomized + 2 caudal regions	20.5
10	Hypophysectomized + 1 cephalic and 1 caudal regions	20.5
11	Hypophysectomized + 1 cephalic, 1 middle and 1 caudal regions	20.5

The brain regions are easily recognized and the head is sufficiently avascular to preclude hemorrhage. Consequently, this stage is best for partial decapitation.

After removal of the vitelline membrane with a tungsten needle (Dossel, 1966), the anterior part of the head from the mid-mesencephalon forward was pinched off and discarded with a pair of forceps. This removed the pituitary morphogenetic field and hypothalamus along with other head derivatives. The window was then resealed with tape and the embryos reincubated.

Grafting

Normal embryos incubated 19 days were donors of anterior pituitary glands. The embryos were killed by decapitation and the sex of each donor was recorded. The head was then cut transversely in half (through the ventral margin of the orbits) exposing the sella turcica, containing the pituitary gland, in the ventral half of the head. The brain and the pars nervosa (not fused with the pars distalis in the chick) were discarded and the pars distalis was stripped of dura mater and transferred with forceps to a depression slide containing a balanced salt solution (0.70 g sodium chloride, 0.042 g potassium chloride and 0.024 g calcium chloride per 100 ml of distilled water; Rugh, 1965). Initially, the pars distalis was observed in

situ and the transfer was made so as to maintain the anterior-posterior orientation which facilitated recognition of the cephalic and caudal regions. This precaution was soon unnecessary because identification of the cephalic and caudal regions became simple (Figure 1).

The gland was then either crushed (in the case of entire pars distalis grafts) or cut into cephalic, middle and caudal regions with a scalpel. The dissecting instruments were rinsed in 70% ethanol after each cut and the separate regions were transferred through two washes of balanced salt solution to minimize cellular contamination between regions before crushing.

Hypophysectomized embryos at 10.5 days of incubation were used as hosts. At this age the chorioallantois readily accepts grafts. The crushed tissue was transferred with forceps through the shell window to a well-vascularized portion of the chorioallantois. As little salt solution as possible was transferred with the tissue to facilitate adhesion to the membrane. The graft type was then recorded on the shell and the host was reincubated.

Killing

At a suitable age (Table 1), the graft was collected for histological examination and the embryo

Figure 1. Regions of chick embryo pars distalis showing where the gland is sectioned to obtain grafts (Courtesy Brasch and Betz, 1971).

was killed by severing the neck immediately below the head. The gross appearance of the embryo was noted as to the abundance and condition of the feathers, the degree of skin cornification, coordination of spontaneous nervous activity, flexure of the toes and body size. In prehatching stages, i.e. 19.5 and 20.5 days of incubation, the degree of yolk sac retraction was noted. Mortality rates were recorded for each group at the time of autopsy. Hypophysectomized embryos were checked for esophageal patency.

All of the extraembryonic membranes were then discarded and the abdomen was opened. The sex was recorded and the duodenum from pylorus to common bile duct was stripped of adherent pancreas and mesenteries, removed, slit lengthwise and rinsed in balanced salt solution (Rugh, 1965). Subsequently, the duodenum was blotted, weighed to the nearest 0.1 mg and placed in a vial in the freezer.

The body weight of the embryo (with head and duodenum removed) was then recorded to the nearest 0.1 g and the length of the right third toe was measured to the nearest mm according to the method of Hamburger and Hamilton (1951). The body was fixed in Bouin's fluid and transferred 24 hours later to 70% ethanol saturated with lithium carbonate. The ethanol and lithium carbonate was changed every 3 or 4 days to

remove excess fixative. The bodies were stored in 70% ethanol.

For histological examination, a 3 mm length of duodenum from the pyloric end and thyroid glands were collected from representative embryos in each group. These tissues were processed for embedding in glycol methacrylate.

Thyroid glands were collected from the fixed bodies, blotted and weighed to the nearest 0.1 mg.

B. Biochemical

Assay of Duodenal Alkaline Phosphatase Activity

'Alkaline Phosphastrate' (65 mM phenolphthalein monophosphate in 7.8 M 2-amino-2-methyl-1-propanol at pH 10.15: Warner-Chilcott Laboratories, Morris Plains, New Jersey) was diluted (2 ml to 25 ml) with glass distilled water to make a working buffered substrate for the assay (Manning et al., 1967).

Glycine buffer, pH 11.2 (Huggins and Talalay, 1945) was prepared by dissolving 9.19 g glycine, 7.19 g sodium chloride and 40 g sodium pyrophosphate in approximately 900 ml of glass distilled water. The pH was adjusted to 11.2 with 30% sodium hydroxide and the solution diluted to 1 l with distilled water. The buffer stopped the enzyme reaction and developed the colour of the phenolphthalein.

Working standards of phenolphthalein were prepared by diluting 0.1, 0.2, 0.3, 0.4 and 0.5 ml respectively of the stock (1 mg/ml in 50% ethanol) to 10 ml with distilled water. Distilled water (0.5 ml) and 0.5 ml of each working standard were transferred to separate test tubes and mixed with 0.5 ml of working buffered substrate. Glycine buffer (2 ml) was then mixed with each solution and absorbances were estimated with a spectrophotometer (Hitachi-Perkin-Elmer, model 139) at 550 m μ . Absorbance was plotted against concentration of phenolphthalein to produce a standard curve (Figure 2). A selected control standard was included with each assay.

The frozen duodenal samples were lyophilized overnight (Virtis 'Unitrap' freeze-dryer) and either stored in the freezer or analyzed immediately. Each sample was homogenized with 2 ml of distilled water in a ground glass homogenizer until it was a uniform dispersion. The volume was adjusted to 3 ml with distilled water. The homogenate was kept at 1°C until incubation with enzyme substrate.

For each experimental group a range of dilutions was prepared to discover a dilution for each reaction, which, after incubation, yielded an absorbance within the linear part of the standard curve. The appropriate homogenate dilution (0.5 ml) was mixed in a

Figure 2. Standard phenolphthalein curve for alkaline phosphatase assay (Manning et al., 1967).

test tube with 0.5 ml of working buffered substrate and the stoppered tube was immediately incubated in a circulating water bath at $37^{\circ} \pm 0.5^{\circ}\text{C}$ until suitable colour intensity developed. The incubation time was recorded and the reaction arrested by adding 2 ml of glycine buffer with immediate agitation. Homogenate blanks for each dilution were obtained by incubating substrate alone and adding 0.5 ml of the appropriate homogenate dilution after the substrate was mixed with the glycine buffer. The sample absorbances were read against the respective blanks at 550 m μ on the spectrophotometer.

Duplicate aliquots of a given homogenate were assayed and the results expressed as an average. The absorbances were converted to phenolphthalein concentration employing a factor derived from the standard curve. These values were multiplied by the dilution factors and incubation time to compute the total enzyme activity in a homogenate as μg phenolphthalein released per hour. Specific activities were calculated by dividing total enzyme activity by corresponding total protein values (Appendix I).

Assay of Total Duodenal Protein

Protein in the duodenum was estimated according to the method of Lowry et al. (1951). Lowry reagent 'D' was prepared immediately prior to use by mixing

50 ml of 2% (w/v) sodium carbonate with 1 ml of Lowry reagent 'B' (0.5% (w/v) copper sulphate pentahydrate in 1% (w/v) sodium potassium tartrate). Lowry reagent 'E' was also made just before use by diluting the Folin-Ciocalteu reagent (Fisher Scientific Co.) with an equal volume of distilled water to yield a 1 N solution.

A solution containing a precisely weighed amount of albumin (Albumin Bovine Fraction V Powder, Sigma Chem. Co., St. Louis) dissolved in 0.5 N sodium hydroxide served as a standard stock, prepared just prior to use and diluted with 0.5 N sodium hydroxide to yield a range of concentrations. These were assayed as described below and the absorbances were plotted versus albumin concentration (Figure 3). A suitable control standard was included each time the assay was performed.

For extraction of protein, 0.5 ml of each homogenate (prepared for the alkaline phosphatase assay) was mixed with 0.5 ml of 10% (w/v) trichloroacetic acid in a centrifuge tube. The tube was then placed in a boiling water bath for 15 minutes to allow precipitation of the proteins. After cooling, each sample was agitated and then centrifuged 10 minutes at 2000 rpm on a Sorval(GLC-1) centrifuge. The supernatant was discarded and the pellet was resuspended in

Figure 3. Standard bovine serum albumin curve for total protein assay (Lowry et al., 1951).

1 ml of 5% (w/v) trichloroacetic acid and centrifuged as before. The second supernatant was discarded and the pellet was dissolved in a volume of 0.5 N sodium hydroxide determined by the dilution required to yield an absorbance in the linear portion of the standard curve.

For the assay, 1 ml of each sample (dissolved in 0.5 N sodium hydroxide) was mixed with 5 ml of Lowry reagent 'D' and allowed to stand 10 minutes. Lowry reagent 'E' (0.5 ml) was then added, with immediate agitation, and the sample was permitted to develop colour for 30 minutes. The sample absorbances were read at 560 mμ on the spectrophotometer. The amount of protein in a given homogenate was calculated by multiplying absorbance times a factor from the standard curve times the dilution factors (Appendix I).

C. Histological

Samples collected for histological examination were fixed at 0°C in 3% (v/v) glutaraldehyde in 0.025 M sodium phosphate buffer ($\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$) at pH 6.8. All dehydration steps were performed at 0-4°C. Following overnight fixation, the tissues were transferred to a wash in 0.025 M phosphate buffer for a duration equivalent to fixation time. Next the tissues were transferred in succession to methyl cellosolve, absolute ethanol, 1-propanol and 1-butanol respectively (two

changes, 12 hours each). After the second 1-butanol change, the tissues were placed in a third change of 1-butanol and permitted to come to room temperature. The butanol was then replaced with glycol methacrylate 'monomer mixture' which was kindly purified (according to the method of Feder, 1965) and supplied by Dr. M. E. McCully of this department. The 'monomer mixture' contained, in addition to purified glycol methacrylate, 0.15% (w/v) α, α - azodiisobutyronitrile (polymerization catalyst) and 5% (w/v) polyethylene glycol 200 (plasticizer) (Feder, 1965). Two further changes of 'monomer mixture' followed the first. Each change was of two days' duration in the dark.

The tissues were then transferred to individual gelatin capsules (size 00) containing fresh 'monomer mixture'. After the tissues were oriented as desired, the capsules were filled to the top with 'monomer mixture', capped and placed in an oven at 60 - 65°C. to polymerize. The polymerized blocks were removed from the oven 24 hours later, trimmed with a jigsaw, and sections were cut from the specimens on a Porter-Blum (Model MT-1) ultramicrotome set at a cutting thickness of 2 μ .

The sections were stained by one of the following procedures:

1. Acid fuchsin and toluidine blue: five minutes in 1% (w/v) aqueous acid fuchsin; five second

distilled water rinse; air dry; 30 seconds to one minute in 0.05% (w/v) toluidine blue in benzoate buffer, pH 4.4 (Sidman et al., 1961); five second distilled water rinse and air dry; mount in Permount.

2. Toluidine blue only: as above.
3. PAS procedure: 20 minute block of free aldehydes in 1% (w/v) sodium chlorite in 1 M acetic acid; 1 hour running tap water rinse; 10 minute oxidation in 0.5% (w/v) periodic acid; 10 minute running tap water rinse; one hour in Schiff reagent (de Tomasi fide Pearse, 1968); three two-minute rinses in separate reducing baths (5 ml of 10% (w/v) potassium metabisulphite + 5 ml of 1 N HCl + 90 ml of distilled water - prepared just prior to use); an additional running tap water rinse (6 minutes); air dry; five seconds in 1% (w/v) aqueous fast green FCF at pH 2.0; a five second distilled water rinse; air dry and mount. Control sections pretreated in 0.01% (w/v) amylase (Sigma Chem. Co., St. Louis), in 0.025 M phosphate buffer, pH 7.0, at $37^{\circ} \pm 0.5^{\circ}\text{C}$, were also treated by the PAS method.

The specimens were examined on a Zeiss Photomicroscope and representative specimens were photographed on Adox KB 14 film.

Three or more specimens were selected at random from each group for histological examination. In the duodenum, mucosal cell heights were measured to the nearest 0.1μ using an ocular micrometer. Three sections from each specimen were randomly selected and ten representative cell heights were determined per section. Numbers and distributions of goblet cells (or their primordia) and of mitotic figures in the mucosal layer were recorded in each of three randomly selected sections per specimen. Staining characteristics were compared from group to group.

Thyroid glands were compared from group to group with respect to relative amounts of tissue components and grafts were compared to normal pituitary histology.

D. Statistics

Group means were calculated for the parameters in Tables 3-10,12,13,15 and 16 and compared statistically using Duncan's new multiple range test (Duncan, 1955) at the 0.05 level of significance or 'Student's' t-test (Bailey, 1959) at the 0.05 or 0.10 level of significance.

RESULTS

General Features

The external features of representative 20.5 day-old embryos from groups 1-5 are shown in Figures 4-8 (Plate I). Embryos in group 2 (hypophysectomized) exhibited a variety of characteristic deficiencies. The feathers were sparser than normal and had curled ends (Compare Figures 4 and 5, Plate I). As late as 17.5 days of incubation, group 2 embryos frequently retained blood-engorged feather germs and feathers were more easily detached during handling than in group 1 (normal).

The skin of group 2 embryos was thinner than in group 1. Blood vessels were readily observed through the skin of the legs and the body was usually pink. The claws of group 2 embryos often detached during handling and the toes were contorted.

Spontaneous nervous activity was feeble, spasmodic and poorly coordinated in group 2 embryos compared to normals.

Normal embryos (group 1) had partially withdrawn their yolk sacs at 19.5 days and almost fully withdrawn them by 20.5 days. There was a concomitant reduction of yolk sac vascularity. Hypophysectomized embryos, however, showed no indication of yolk sac withdrawal

or vascular reduction even by 20.5 days.

In groups 3 and 4 (hypophysectomized + a cephalic region graft and hypophysectomized + a middle region graft) these deficiencies were repaired (Compare Figures 4-7, Plate I). Skin and feathers looked normal, twisting of toes was seldom seen, and spontaneous nervous activity was similar to that of group 1 embryos. Group 5 embryos (hypophysectomized + a caudal region graft) appeared no different from hypophysectomized embryos (group 2) (Compare Figures 5 and 8, Plate I).

Mortality rates for groups 1-5 embryos are presented in Table 2. In group 1, mortality rate was constant from 16.5 to 19.5 days, increasing at 20.5 days. The 15.5 day-old class had an unusually high death rate. Group 2 mortality rates similarly increased with age but mortality was much higher at all ages except 15.5 days. Groups 3-5 embryos survived better than group 2 embryos and groups 3 and 5 displayed the same tendency to have increased mortality in older classes. Group 4 embryos did not show this trend.

All hypophysectomized embryos (including the grafted groups) of course lacked the anterior brain regions, eyes and upper beak, all of which were removed along with the pituitary by partial decapitation (See Figure 9, Plate I). In most cases, these embryos possessed a lower beak and an open (patent) esophagus but some operated embryos (37%) also lacked these

PLATE I

External features of embryos from groups 1-5 at 20.5 days of incubation. Scale line (Figure 4) represents 1.5 cm in Figures 4-10.

Figure 4. Headless body of normal embryo.

Figure 5. Headless body of hypophysectomized embryo. Note reduced body size, clenched toes and curly feathers.

Figure 6. Headless body of hypophysectomized embryo which received one cephalic region pars distalis graft. Compare with Figures 4 and 5.

Figure 7. Headless body of hypophysectomized embryo which received one middle region pars distalis graft. Compare with Figure 6.

Figure 8. Headless body of hypophysectomized embryo which received one caudal region pars distalis graft. Compare with Figure 5.

Figure 9. Head of hypophysectomized embryo with a patent esophagus (large arrow). Small arrow indicates auditory meatus.

Figure 10. Head of hypophysectomized embryo with a non-patent esophagus. Opening (arrow) is auditory meatus.

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TABLE 2 PERCENT MORTALITY

Group	Days of Incubation					
	15.5 ..	16.5	17.5	18.5	19.5	20.5
1. Normal	47(15)*	15(13)	11(28)	11(41)	14(35)	23(47)
2. Hypophysectomized	39(23)	46(39)	67(51)	75(52)	82(129)	89(138)
3. Hypophysectomized + cephalic region graft	-	-	30(20)	39(18)	54(37)	52(27)
4. Hypophysectomized + middle region graft	-	-	53(19)	35(17)	29(24)	43(21)
5. Hypophysectomized + caudal region graft	-	-	35(17)	50(20)	54(35)	58(19)

* Numbers in parentheses represent number of embryos started for each group.

features (non-patent esophagus) (Compare Figures 9 and 10, Plate I). The significance of esophageal patency is examined in a later section.

Body Growth

Body growth assessed by body weight shows significant daily increments between 16.5 days and 19.5 days in group 1 (Table 3). Body weight also increases, although not significantly, between 15.5 and 16.5 days and between 19.5 and 20.5 days in this group. Group 2 embryos had a body growth pattern similar to group 1 in terms of body weight, except that significant daily increments extended from 15.5 through 19.5 days. Grafted groups (3-5) exhibited body growth patterns different from normal with significant increases over a 24-hour period occurring less frequently. The patterns of body weight increases for groups 1-5 are summarized in Table 3 and Figure 11.

Third toe length increases in group 1 follow a pattern similar to that for body weight except for significant growth of the third toe between 15.5 and 16.5 days (Table 3, Figure 12). The pattern of third toe growth in group 2 is slightly different from normal apparently slowing between 16.5 and 17.5 days as well as between 18.5 days and 19.5 days. In groups 3-5 the pattern of toe growth is again different from normal. There is a tendency for significant daily

TABLE 3 FRESH WEIGHT (g) OF HEADLESS BODIES AND LENGTH (mm) OF RIGHT THIRD TOE

Group	Parameter	Days of Incubation					
		15.5	16.5	17.5	18.5	19.5	20.5
Normal	Body wt.	7.3±0.2* (8)+	9.4±0.2 (11)	11.4±0.3 (23)	14.7±0.4 (25)	18.4±0.6 (12)	19.2±0.8 (26)
	Toe length	14±0.5 (8)	17±0.3 (11)	19±0.2 (25)	22±0.2 (25)	23±0.3 (13)	23±0.2 (27)
Hypophysectomized	Body wt.	5.7±0.2 (12)	7.5±0.2 (14)	8.9±0.3 (18)	11.0±0.5 (11)	13.2±0.5 (17)	13.8±0.5 (11)
	Toe length	12±0.2 (14)	14±0.2 (14)	15±0.4 (18)	17±0.4 (11)	18±0.2 (17)	20±0.3 (11)
Hypophysectomized + cephalic region graft	Body wt.	-	-	11.4±0.5 (10)	11.6±0.9 (11)	14.7±0.9 (13)	16.2±1.1 (12)
	Toe length	-	-	18±0.3 (10)	19±0.6 (11)	21±0.5 (13)	23±0.5 (12)
Hypophysectomized + middle region graft	Body wt.	-	-	10.1±0.4 (7)	13.8±0.8 (10)	14.9±0.6 (13)	15.2±0.7 (12)
	Toe length	-	-	18±0.5 (7)	20±0.8 (11)	22±0.5 (13)	23±0.5 (12)
Hypophysectomized + caudal region graft	Body wt.	-	-	9.9±0.4 (12)	9.7±0.8 (8)	11.3±0.6 (9)	11.9±0.8 (10)
	Toe length	-	-	16±0.4 (12)	16±0.4 (8)	17±0.4 (9)	19±0.6 (10)

Mean ± S.E. + # of samples x Values underscored by lines at the same level are not significantly different.

TABLE 4 COMPARISON OF GROUP MEANS IN TABLE 3
BY DUNCAN'S NEW MULTIPLE RANGE TEST

Days of Incubation	Parameter	Group				
15.5	Body weight	1*	2	-	-	-
	Toe length	1	2	-	-	-
16.5	Body weight	1	2	-	-	-
	Toe length	1	2	-	-	-
17.5	Body weight	x <u>1</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>2</u>
	Toe length	<u>1</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>2</u>
18.5	Body weight	<u>1</u>	<u>4</u>	<u>3</u>	<u>2</u>	<u>5</u>
	Toe length	1	<u>4</u>	<u>3</u>	<u>2</u>	<u>5</u>
19.5	Body weight	1	<u>4</u>	<u>3</u>	<u>2</u>	<u>5</u>
	Toe length	<u>1</u>	<u>4</u>	<u>3</u>	<u>2</u>	<u>5</u>
20.5	Body weight	1	<u>3</u>	<u>4</u>	<u>2</u>	<u>5</u>
	Toe length	<u>1</u>	<u>3</u>	<u>4</u>	<u>2</u>	<u>5</u>

* Groups are arranged in descending order with respect to their means in Table 3.

x Values underscored by lines at the same level are not significantly different.

Figure 11. Graph of daily changes in the weight of the headless fresh bodies in groups 1($\square$), 2($\triangle$), 3($\circ$), 4($\blacktriangle$) and 5($\bullet$).

Figure 12. Graph of daily change in the length of the right third toe of embryos in groups 1($\square$), 2($\triangle$), 3($\circ$), 4($\blacktriangle$) and 5($\oplus$).

growth to occur later than in group 1, i.e. between 18.5 and 20.5 days.

Body growth is significantly subnormal in group 2 from 15.5 through 20.5 days on the basis of either body weight or toe length (See Tables 3 and 4, Figures 11 and 12). Hypophysectomized embryos with cephalic region or middle region grafts exhibited a tendency to improve body growth (body weight and/or toe length) toward the normal level although the results for toe length are more compelling than those for body weight. In no case was body growth in group 5 any better than for hypophysectomized embryos (group 2).

Duodenal Growth

The day-to-day changes in duodenal weight tend to increase in all groups (Table 5, Figure 13). Significant daily increments appear between 17.5 and 18.5 days in normal embryos, a day later in the absence of the pars distalis and not until 20.5 days in the grafted groups. Significant growth occurs between 15.5 and 16.5 days in group 2. The changes in relative duodenal weight are smaller and significant increases over a 24-hour period are less frequent in all cases except group 4 (Figure 14). The pattern of relative growth in group 2 (no pars distalis) is disturbed. Probably no treatment completely restored the normal kinetics of the daily growth pattern.

TABLE 5 DUODENAL FRESH WEIGHT (mg) AND FRESH WEIGHT RELATIVE TO BODY WEIGHT (mg/g)

Group	Parameter	Days of Incubation				
		15.5	16.5	17.5	18.5	20.5
1. Normal	Fresh wt.	20.8±0.9* (8)†	28.6±0.8 (11)	34.3±1.0 (25)	50.8±1.2 (25)	65.4±2.3 (14)
	x					75.2±3.4 (27)
	$\frac{\text{Fr. wt.}}{\text{Body wt.}}$	2.9±0.1 (8)	3.0±0.1 (11)	3.0±0.1 (23)	3.5±0.1 (25)	3.6±0.1 (12)
2. Hypophysectomized	Fresh wt.	14.9±0.7 (14)	19.5±0.7 (14)	22.4±0.8 (18)	25.7±1.7 (11)	33.2±2.1 (17)
	$\frac{\text{Fr. wt.}}{\text{Body wt.}}$	2.6±0.1 (12)	2.6±0.1 (14)	2.6±0.1 (17)	2.3±0.1 (11)	2.5±0.1 (17)
						2.9±0.1 (11)
3. Hypophysectomized + cephalic region graft	Fresh wt.	-	-	34.0±1.9 (10)	39.7±3.7 (11)	47.8±2.5 (13)
	$\frac{\text{Fr. wt.}}{\text{Body wt.}}$	-	-	3.0±0.2 (10)	3.4±0.2 (11)	3.3±0.1 (13)
						3.7±0.3 (12)
4. Hypophysectomized + middle region graft	Fresh wt.	-	-	26.6±0.7 (7)	44.2±2.8 (11)	48.9±0.3 (13)
	$\frac{\text{Fr. wt.}}{\text{Body wt.}}$	-	-	2.7±0.1 (7)	3.3±0.3 (10)	3.3±0.1 (13)
						3.9±0.1 (12)
5. Hypophysectomized + caudal region graft	Fresh wt.	-	-	23.6±0.9 (12)	26.6±1.6 (8)	29.9±0.8 (9)
	$\frac{\text{Fr. wt.}}{\text{Body wt.}}$	-	-	2.4±0.1 (12)	2.8±0.2 (8)	2.7±0.2 (9)
						3.2±0.1 (10)

* Mean ± S.E. + # of samples x Values underscored by lines at the same level are not significantly different.

TABLE 6 COMPARISON OF GROUP MEANS IN TABLE 5
BY DUNCAN'S NEW MULTIPLE RANGE TEST

Days of Incubation	Parameter	Group				
15.5	Fresh Weight	x <u>1*</u>	<u>2</u>	-	-	-
	<u>Fr. wt.</u>	<u>1</u>	<u>2</u>	-	-	-
	<u>Body wt.</u>					
16.5	Fresh Weight	1	2	-	-	-
	<u>Fr. wt.</u>	1	2	-	-	-
	<u>Body wt.</u>					
17.5	Fresh Weight	<u>1</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>2</u>
	<u>Fr. wt.</u>	<u>1</u>	<u>3</u>	<u>4</u>	<u>2</u>	<u>5</u>
	<u>Body wt.</u>					
18.5	Fresh Weight	1	<u>4</u>	<u>3</u>	<u>5</u>	<u>2</u>
	<u>Fr. wt.</u>	<u>1</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>2</u>
	<u>Body wt.</u>					
19.5	Fresh Weight	1	<u>4</u>	<u>3</u>	<u>2</u>	<u>5</u>
	<u>Fr. wt.</u>	1	<u>3</u>	<u>4</u>	<u>5</u>	<u>2</u>
	<u>Body wt.</u>					
20.5	Fresh Weight	1	<u>4</u>	<u>3</u>	<u>2</u>	<u>5</u>
	<u>Fr. wt.</u>	<u>4</u>	<u>1</u>	<u>3</u>	<u>5</u>	<u>2</u>
	<u>Body wt.</u>					

* Groups are arranged in descending order with respect to their means in Table 5.

x Values underscored by lines at the same level are not significantly different.

Figure 13. Graph of daily changes in the weight of fresh duodena of embryos in groups 1($\square$), 2($\triangle$), 3($\circ$), 4($\blacktriangle$) and 5($\oplus$).

Figure 14. Graph of daily changes in fresh duodenal weight relative to fresh body weight of embryos in groups 1($\square$), 2($\triangle$), 3($\circ$), 4($\blacktriangle$) and 5($\odot$).

Duodenal growth in the absence of the pars distalis is significantly subnormal from 16.5 days onward on both an absolute and relative basis (Table 6). Cephalic grafts (group 3) restore duodenal weight to normal at 17.5 days, but thereafter repair of this parameter is incomplete although significantly improved as compared to the arrested duodenal growth in hypophysectomized embryos (group 2). Middle grafts (group 4) effect less repair than cephalic grafts at 17.5 days, but are not different thereafter. Caudal grafts (group 5) were incapable of increasing duodenal weights over the levels for hypophysectomized embryos. The magnitude of the changes between treatment groups is generally the same for the relative and absolute duodenal weights (Table 6). However, relatively groups 3 and 4 were not different from group 5 at 18.5 days and group 3 was not significantly better than group 5 at 20.5 days. But, at 19.5 days the significant differences are the same in either absolute or relative terms. During the last three days, duodenal growth is not restored to normal by cephalic or middle grafts but it is significantly improved as compared to the hypophysectomized group or to those with caudal grafts, a treatment which did not produce any significant growth.

Duodenal growth, assessed by total protein content (Table 7, Figure 15) again tends to increase in all groups although the differences are less striking than

TABLE 7 DUODENAL TOTAL PROTEIN (mg) AND PROTEIN RELATIVE TO DUODENAL FRESH WEIGHT (mg/g)

Group	Parameter	Days of Incubation					
		15.5	16.5	17.5	18.5	19.5	20.5
1. Normal	Total Protein	1.0±0.1* (8)+	2.2±0.1 (11)	2.7±0.1 (24)	4.5±0.1 (24)	4.6±0.2 (14)	5.3±0.2 (27)
	<u>Protein</u> <u>Duodenal wt.</u>	69±2 (8)	85±2 (9)	82±2 (24)	88±2 (24)	78±2 (14)	75±2 (27)
2. Hypophysectomized	Total Protein	0.5±0.03 (14)	1.2±0.1 (14)	1.8±0.1 (19)	1.8±0.1 (11)	2.5±0.2 (17)	2.7±0.2 (11)
	<u>Protein</u> <u>Duodenal wt.</u>	63±4 (14)	80±3 (14)	85±1 (19)	75±4 (11)	77±2 (17)	70±4 (11)
3. Hypophysectomized + cephalic region graft	Total Protein	-	-	2.6±0.1 (10)	3.1±0.3 (11)	3.6±0.2 (13)	4.5±0.5 (12)
	<u>Protein</u> <u>Duodenal wt.</u>	-	-	85±2 (10)	84±3 (11)	79±2 (13)	81±3 (12)
4. Hypophysectomized + middle region graft	Total Protein	-	-	2.1±0.1 (7)	3.2±0.2 (11)	3.9±0.3 (13)	4.4±0.2 (12)
	<u>Protein</u> <u>Duodenal wt.</u>	-	-	89±5 (7)	79±2 (11)	84±1 (13)	78±3 (12)
5. Hypophysectomized + caudal region graft	Total Protein	-	-	1.7±0.1 (12)	2.2±0.2 (8)	2.3±0.1 (9)	2.6±0.2 (10)
	<u>Protein</u> <u>Duodenal wt.</u>	-	-	78±2 (12)	88±4 (8)	79±3 (9)	72±3 (10)

Mean ± S.E. + # of samples

x Values underscored by lines at the same level are not significantly different.

TABLE 8 COMPARISON OF GROUP MEANS IN TABLE 7
BY DUNCAN'S NEW MULTIPLE RANGE TEST

Days of Incubation	Parameter	Group				
15.5	Total Protein	1*	2	-	-	-
	$\frac{\text{Protein}}{\text{Duodenal wt.}}$	x <u>1</u>	<u>2</u>	-	-	-
16.5	Total Protein	1	2	-	-	-
	$\frac{\text{Protein}}{\text{Duodenal wt.}}$	<u>1</u>	<u>2</u>	-	-	-
17.5	Total Protein	<u>1</u>	<u>3</u>	<u>4</u>	<u>2</u>	<u>5</u>
	$\frac{\text{Protein}}{\text{Duodenal wt.}}$	<u>4</u>	<u>3</u>	<u>2</u>	<u>1</u>	<u>5</u>
18.5	Total Protein	1	<u>4</u>	<u>3</u>	<u>5</u>	<u>2</u>
	$\frac{\text{Protein}}{\text{Duodenal wt.}}$	<u>5</u>	<u>1</u>	<u>3</u>	<u>4</u>	<u>2</u>
19.5	Total Protein	1	<u>4</u>	<u>3</u>	<u>2</u>	<u>5</u>
	$\frac{\text{Protein}}{\text{Duodenal wt.}}$	<u>4</u>	<u>5</u>	<u>3</u>	<u>1</u>	<u>2</u>
20.5	Total Protein	1	<u>3</u>	<u>4</u>	<u>2</u>	<u>5</u>
	$\frac{\text{Protein}}{\text{Duodenal wt.}}$	<u>3</u>	<u>4</u>	<u>1</u>	<u>5</u>	<u>2</u>

* Groups are arranged in descending order with respect to their means in Table 7.

x Values underscored by lines at the same level are not significantly different.

Figure 15. Graph of daily changes in total duodenal protein of embryos in groups 1($\square$), 2($\triangle$), 3($\circ$), 4($\blacktriangle$) and 5($\bullet$).

the weight changes. Significant daily increments are less frequent except in group 4. Protein content expressed relative to duodenal weight erases the increasing trends (Table 7, Figure 16). The data show considerable fluctuation and the only general pattern is a possible peak between 16.5 and 18.5 days and a decline by 20.5 days. Duodenal total protein is subnormal in the absence of the pars distalis (group 2) throughout the period studied (Table 8). The differences between the treatment groups for total protein are remarkably similar to those for the weight data, except at 17.5 days, growth with middle, caudal or no grafts is subnormal to the same degree. Generally, cephalic and middle grafts significantly improve duodenal protein content (although not to normal levels) but caudal grafts are unsuccessful in raising protein content above the level in hypophysectomized embryos.

The developmental pattern of duodenal alkaline phosphatase activity (Table 9. Figures 17 and 18) is almost the same whether expressed relative to protein content (specific activity) or to duodenal weight. The trends are generally consistent with those in the protein content and weight data. It should be noted however that the increases in groups 2 and 5 are slight and significant changes appear only during the final two days of incubation. Despite the dramatic increases the significant daily increments are limited to the 20.5-day values in groups 1 and 3. Throughout the study

Figure 16. Graph of daily changes in total duodenal protein relative to fresh duodenal weight of embryos in groups 1($\square$), 2($\triangle$), 3($\circ$), 4($\blacktriangle$) and 5($\odot$).

TABLE 9 DUODENAL ALKALINE PHOSPHATASE SPECIFIC ACTIVITIES RELATIVE TO PROTEIN ($\mu\text{g/hr}$)
(mg)
AND RELATIVE TO DUODENAL FRESH WEIGHT ($\mu\text{g/hr}$)
(mg)

Group	Parameter	Days of Incubation					
		15.5	16.5	17.5	18.5	19.5	20.5
Normal	Activity Protein	387 $\pm$ 14* (8) ⁺	435 $\pm$ 19 (11)	901 $\pm$ 83 (24)	1,810 $\pm$ 150 (24)	2,856 $\pm$ 383 (14)	6,535 $\pm$ 704 (27)
	Activity Fr. wt.	27 $\pm$ 1 (8)	38 $\pm$ 2 (9)	73 $\pm$ 7 (24)	160 $\pm$ 14 (24)	219 $\pm$ 27 (14)	493 $\pm$ 56 (27)
	Activity Protein	327 $\pm$ 32 (14)	311 $\pm$ 23 (14)	313 $\pm$ 19 (19)	279 $\pm$ 25 (11)	381 $\pm$ 34 (17)	470 $\pm$ 68 (11)
Hypophysectomized	Activity Fr. wt.	20 $\pm$ 2 (14)	24 $\pm$ 2 (14)	27 $\pm$ 2 (18)	22 $\pm$ 3 (11)	29 $\pm$ 2 (17)	31 $\pm$ 3 (11)
	Activity Protein	-	-	760 $\pm$ 199 (10)	2,089 $\pm$ 440 (11)	2,036 $\pm$ 506 (13)	5,089 $\pm$ 1305 (12)
	Activity Fr. wt.	-	-	67 $\pm$ 19 (10)	177 $\pm$ 36 (11)	162 $\pm$ 41 (13)	415 $\pm$ 104 (12)
Hypophysectomized + middle region graft	Activity Protein	-	-	661 $\pm$ 151 (7)	1,947 $\pm$ 380 (11)	2,015 $\pm$ 505 (13)	3,308 $\pm$ 879 (12)
	Activity Fr. wt.	-	-	58 $\pm$ 14 (7)	158 $\pm$ 33 (11)	171 $\pm$ 43 (13)	249 $\pm$ 61 (12)
	Activity Protein	-	-	326 $\pm$ 23 (12)	354 $\pm$ 44 (8)	536 $\pm$ 113 (9)	400 $\pm$ 33 (10)
Hypophysectomized + caudal region graft	Activity Fr. wt.	-	-	26 $\pm$ 2 (12)	31 $\pm$ 4 (8)	42 $\pm$ 9 (9)	28 $\pm$ 2 (10)

Mean $\pm$ S.E. + # of samples

x Values underscored by lines at the same level are not significantly different.

TABLE 10 COMPARISON OF GROUP MEANS IN TABLE 9
BY DUNCAN'S NEW MULTIPLE RANGE TEST

Days of Incubation	Parameter	Group				
15.5	Activity Protein	x <u>1*</u>	<u>2</u>	-	-	-
	Activity Fr. wt.	1	2	-	-	-
16.5	Activity Protein	1	2	-	-	-
	Activity Fr. wt.	1	2	-	-	-
17.5	Activity Protein	<u>1</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>2</u>
	Activity Fr. wt.	<u>1</u>	<u>3</u>	<u>4</u>	<u>2</u>	<u>5</u>
18.5	Activity Protein	<u>3</u>	<u>4</u>	<u>1</u>	<u>5</u>	<u>2</u>
	Activity Fr. wt.	<u>3</u>	<u>1</u>	<u>4</u>	<u>5</u>	<u>2</u>
19.5	Activity Protein	<u>1</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>2</u>
	Activity Fr. wt.	<u>1</u>	<u>4</u>	<u>3</u>	<u>5</u>	<u>2</u>
20.5	Activity Protein	<u>1</u>	<u>3</u>	<u>4</u>	<u>2</u>	<u>5</u>
	Activity Fr. wt.	<u>1</u>	<u>3</u>	<u>4</u>	<u>2</u>	<u>5</u>

* Groups are arranged in descending order with respect to their means in Table 9.

x Values underscored by lines at the same level are not significantly different.

Figure 17. Graph of daily changes in alkaline phosphatase specific activity relative to total duodenal protein of embryos in groups 1($\square$), 2($\triangle$), 3($\circ$), 4($\blacktriangle$) and 5($\bullet$).

Figure 18. Graph of daily changes in alkaline phosphatase specific activity relative to fresh duodenal weight of embryos in groups 1($\square$), 2($\triangle$), 3($\circ$), 4($\blacktriangle$) and 5($\bullet$).

cephalic and middle grafts restored normal alkaline phosphatase levels (Table 10) except for the subnormal middle graft value at 20.5 days. Nevertheless, group 4 embryos always had significantly higher duodenal phosphatase activities than those for hypophysectomized embryos which were subnormal throughout (except relative to duodenal protein at 15.5 days). The chorioallantoic grafts from 19-day old donors implanted into 10.5-day old hosts begin to affect duodenal development before 17.5 days of incubation and near normal levels of development are quickly attained.

Duodenal Histology

The histology of the duodena from groups 1-5 is illustrated in Figures 19-49 (Plates II-VI) and summarized in Table 11.

The duodenum of group 1 embryos at 15.5 days (Figure 19) has relatively short villi with a loose stroma (lamina propria), frequently containing erythrocyte aggregations. The stroma occupies a large portion of the villus. The epithelium is cuboidal or low columnar with intensely basophilic cytoplasm (Table 11). The epithelial cells often have perinuclear vacuoles. No brush border is apparent at the luminal surface. Most goblet cell primordia are confined to the bases of the villi but mitotic figures occur throughout the epithelium (Table 11).

TABLE 11 SUMMARY OF DUODENAL HISTOLOGY

Days of Incubation	Group	Mucosal Cell Ht. (μ)	Cell Ht. Width	Goblet Cells*	Mitotic Figures*	PAS-positive Brush Border	PAS-positive Granules	Basophilia†
15.5	1	12.6	1.7	15(79)	15(36)	-	-	+++
	2	8.4	1.6	0	19(37)	-	-	+++
16.5	1	11.0	1.7	16(60)	27(37)	-	-	+++
	2	11.1	1.9	11(61)	14(37)	-	-	+++
17.5	1	11.3	2.4	24(51)	22(49)	trace	++	+++
	2	14.9	2.7	5(69)	17(35)	0	++	+++
	3	10.9	1.9	25(56)	45(35)	trace	+	+++
	4	12.5	2.2	24(55)	42(40)	0	++	+++
	5	10.1	2.1	12(52)	19(42)	0	++	+++
18.5	1	18.5	3.1	77(42)	40(47)	+	+	++
	2	9.6	2.1	12(64)	20(45)	0	++	+++
	3	18.6	3.4	87(49)	38(60)	+	+	+
	4	18.4	3.4	146(47)	42(47)	+	+	++
	5	13.7	2.8	5(60)	16(46)	0	++	+++
19.5	1	29.3	5.3	189(43)	43(66)	++	trace	+
	2	14.3	2.4	21(58)	21(44)	0	++	+++
	3	26.1	4.3	174(42)	45(58)	++	+	+
	4	23.8	3.7	128(34)	39(58)	++	+	+
	5	13.3	2.5	38(60)	39(56)	0	++	+++
20.5	1	34.9	6.4	357(37)	56(63)	+++	0	0-+
	2	17.2	2.9	25(76)	41(54)	0	++	+++
	3	40.2	7.1	417(42)	26(67)	+++	0	0
	4	27.4	5.8	343(31)	38(65)	+++	trace	0
	5	12.8	1.9	28(72)	34(37)	0	++	+++

* Table values represent total # per cross-section and percentage of total (in parentheses) confined to base of villus.

† Values 0 to +++ represent relative amounts of stainable material in the mucosal cell cytoplasm. PAS technique was not applied to 15.5 or 16.5 day groups.

Even by 17.5 days in group 1 duodena, the morphology has not changed appreciably (Table 11 and Figures 21 and 23, Plate II). There is apparently a slight increase in villous length and duodenal diameter. The epithelial cell cytoplasm contains abundant PAS-positive granules and the luminal border begins to be faintly PAS-positive (visible only when villi were apposed). (The earliest stage treated by the PAS technique was 17.5 days.) The mucosal cells are narrower but not taller. Goblet cell primordia have increased slightly in frequency and their distribution is less confined to the bases of the villi. Conversely, a greater proportion of mitotic figures are located at the villous bases (Table 11).

From 18.5 days to 20.5 days in group 1 duodena there is a progressive increase in villous length. The stroma becomes more compact and occupies a much smaller proportion of the villus (Figures 23, Plate II and 38, Plate V). The columnar epithelial cells increase remarkably in height by 20.5 days (Table 11 and Figures 28, Plate III; 33, Plate IV and 38 and 39, Plate V). During the same period the intensity of the basophilia and number of PAS-positive granules gradually decline and the granules are absent by 20.5 days. The large perinuclear vacuoles have also disappeared by 20.5 days. The brush border becomes thicker and is strongly PAS-positive by 20.5 days (Table 11 and Figure 44, Plate VI). Goblet cells

increase in number and are spread throughout the epithelium whereas mitotic figures tend to become localized at the bases of the villi and in the crypts of Lieberkühn by 20.5 days.

The duodena of group 2 embryos do not look different from group 1 duodena until 18.5 days (Compare Figures 19-24, Plate II). From 18.5 to 20.5 days, morphogenesis of group 2 duodena is arrested or retarded compared to group 1 (Compare Figures 28 and 29, Plate III; Figures 33 and 34, Plate IV; and Figures 38-40, Plate V). The villous length and mucosal cell height do not increase appreciably (Table 11). Cytoplasmic basophilia and PAS-positive granules persist even at 20.5 days (Figures 40, Plate V and 45, Plate VI). The granules were only slightly depleted by amylase digestion although they appeared more finely distributed after this treatment (Compare Figures 45 and 46, Plate VI). Perinuclear vacuoles are still present at 20.5 days. A brush border is not apparent in group 2 duodena. The numbers and distribution of goblet cells show no striking changes after 18.5 days and mitotic figures exhibit only a slight tendency to be confined to the villous bases (Table 11).

In groups 3 and 4, duodena at 17.5 days do not appear to be different from those of groups 1 and 2 (Compare Figures 23 and 24, Plate II and Figures 25 and 26, Plate III). From 18.5 to 20.5 days, however, the

level of morphogenesis of the duodena in groups 3 and 4 is within the normal range and is improved over the duodena of hypophysectomized (group 2) embryos (Compare Figures 28-30, Plate III and Figure 31, Plate IV; Figures 33-36, Plate IV; Figures 38-42, Plate V and Figures 44-48, Plate VI). Compare also groups 1-4 from 18.5-20.5 days in Table 11.

The duodena of the embryos in group 5 show no appreciable differences from those of groups 1-4 at 17.5 days (Table 11 and Figures 23 and 24, Plate II and Figures 25-27, Plate III). From 18.5 to 20.5 days in group 5 the pattern of duodenal morphogenesis follows closely that in group 2 and is distinctly subnormal (Compare Figures 29, Plate III and 32, Plate IV; Figures 34, Plate IV and 37, Plate V; Figures 40, Plate V and 43, Plate VI and Figures 45 and 49, Plate VI).

Thus, during the final three days of incubation, the pattern of duodenal morphogenesis in group 2 was arrested or retarded as compared to normal, at least according to the developmental parameters examined here. However, duodenal morphogenesis in the embryos in groups 3 and 4 was within the normal (group 1) range. In group 5 duodenal morphogenesis did not exceed the retarded level in group 2.

Duodenal Growth and Differentiation in Ancillary Groups

The fresh weights, total protein content and alkaline phosphatase specific activities for the duodena of ancillary groups are presented in Table 12 and these group means are compared with the means from groups 1-5 at 20.5 days in Table 13. These developmental parameters were chosen because they showed the greatest differences between groups.

In terms of duodenal fresh weight, no group at 20.5 days was restored to the normal level (Table 13). Groups 3, 4 and 6 were significantly greater than group 2. No other group was significantly different from group 2. Groups 7, 8 and 10 were not significantly less than groups 3, 4 and 6 and group 11 was not significantly lower than groups 3, 4, 7, 8 and 10 in terms of duodenal fresh weight.

On the basis of duodenal total protein, graft performance was similar to that indicated by the fresh weight data (Tables 12 and 13). However, only groups 5, 9 and 11 had total protein values within the least significant range of the level for group 2 but group 11 again was not significantly lower than groups 3, 4, 6, 7, 8 and 10. Groups 3, 4, 6-8 and 10 attained levels of duodenal total protein within the normal range.

Duodenal alkaline phosphatase specific activity was restored to the normal level in groups 3, 6, 7 and 8. Groups 4, 5 and 9-11 failed to attain a significantly

TABLE 12 FRESH WEIGHT (mg), TOTAL PROTEIN (mg) AND SPECIFIC ACTIVITY OF ALKALINE PHOSPHATASE ($\frac{\mu\text{g/hr}}{\text{mg}}$) FOR THE DUODENA OF ANCILLARY GROUPS

Parameter	Group				
	6	7	8	9	10
Fresh Weight	61.8 $\pm$ 7.6* (9) $\frac{1}{2}$	53.1 $\pm$ 6.1 (10)	51.9 $\pm$ 4.8 (8)	41.0 $\pm$ 4.2 (9)	51.8 $\pm$ 5.4 (6)
Total Protein	5.1 $\pm$ 0.6 (9)	4.7 $\pm$ 0.5 (10)	4.5 $\pm$ 0.3 (8)	3.2 $\pm$ 0.2 (9)	4.4 $\pm$ 0.5 (6)
Alkaline Phosphatase (Activity/Protein)	7727 $\pm$ 1198 (9)	6252 $\pm$ 1660 (10)	4216 $\pm$ 1803 (8)	632 $\pm$ 204 (8)	2864 $\pm$ 877 (6)
					1864 $\pm$ 580 (6)

* Mean $\pm$ S.E. * # of samples

TABLE 13 COMPARISON OF GROUP MEANS IN TABLE 12 WITH THE MEANS OF GROUPS 1-5 (AT 20.5 DAYS) FROM TABLES 5, 7 AND 9 BY DUNCAN'S NEW MULTIPLE RANGE TEST

Parameter	Group									
Fresh Weight	1*	6	4	3	7	8	10	11	9	2 5
	x									
Total Protein	1	6	7	3	8	4	10	11	9	2 5
Alkaline Phosphatase (Activity/Protein)	6	1	7	3	8	4	10	11	9	2 5

* Groups are arranged in descending order with respect to their means in Tables 5, 7, 9 and 12.

x Values underscored by lines at the same level are not significantly different.

improved level of specific activity as compared to group 2. However, groups 4 and 10 had intermediate values which were not different from groups 3, 7 and 8 and alkaline phosphatase specific activity in group 11 was not significantly lower than groups 3, 4, 8 and 10 (Table 13).

In general, all grafts containing cephalic region pars distalis tissue (groups 3, 4, 6-8, 10 and 11) achieved intermediate or normal levels of duodenal growth and differentiation but some of these groups were not significantly better than group 2. In particular, groups 10 and 11 were consistently the least effective treatments in which cephalic region pars distalis tissue was present in the graft. In no case did groups 5 and 9 effect any repair. Moreover, it is evident that grafts of 2 cephalic (group 7) or 2 middle (group 8) or 2 caudal regions (group 9), respectively, were no more effective than single grafts of the corresponding regions (Table 13).

Duodenal Histology in Ancillary Groups

The histology of the duodena from groups 6-11 is illustrated in Figures 50 and 51, Plate VI; Figures 52-58, Plate VII and Figures 59-62, Plate VIII, and summarized in Table 14.

In groups 6-8 (Figures 50 and 51, Plate VI; Figures 53, 57 and 58, Plate VII and Figure 59, Plate VIII)

TABLE 14 SUMMARY OF DUODENAL HISTOLOGY OF ANCILLARY GROUPS

Group	Mucosal Cell Ht. (μ)	Cell Ht. Cell Width	Goblet* Cells	Mitotic* Figures	PAS-positive Brush Border	PAS-positive* Granules	Basophilic†
6	31.0	5.6	182(33)	35(78)	+++	+	+
7	30.1	5.6	388(41)	54(78)	+++	0	+
8	25.2	4.0	432(30)	55(70)	+++	trace	+
9	11.5	1.8	53(70)	54(32)	trace	++	+++
10	18.6	3.2	114(41)	44(37)	++	+	++
11	21.2	4.4	350(34)	52(41)	+++	0	++

Table values represent total # per cross-section and percentage of total (in parentheses) confined to base of villus.

Values 0 to +++ represent relative amounts of stainable material in the mucosal cell cytoplasm.

duodenal morphogenesis attained a level within the normal range at 20.5 days (Figures 38 and 39, Plate V and Figure 44, Plate VI). The villi in groups 6 and 8 did not appear as elongated nor was the stroma as compact or reduced as in the normal duodenum at 20.5 days. In addition, the numbers of goblet cells were reduced in group 6 (Tables 11 and 14).

In groups 10 and 11 (Figures 55 and 56, Plate VII and Figures 61 and 62, Plate VIII) duodenal morphology was subnormal (Compare with Figures 38 and 39, Plate V and Figure 44, Plate VI) but apparently improved over group 2 (Figure 40, Plate V and Figure 45, Plate VI). In particular, the epithelial cells were not as tall as normal and their cytoplasm was more strongly basophilic. Goblet cell numbers were reduced in group 10 but the distribution appeared normal. Mitotic figures were not localized at the bases of the villi in duodena of groups 10 and 11 but in other respects, the duodena were normal (Tables 11 and 14).

In group 9 (Figure 54, Plate VII and Figure 60, Plate VIII) duodenal morphogenesis was retarded or arrested at the same level as group 2 (Figure 40, Plate V and Figure 45, Plate VI; Tables 11 and 14).

Thus, duodenal morphology was repaired substantially in groups 6-8, moderately in groups 10 and 11, but not at all in group 9.

Effect of Esophageal Patency on Duodenal Development

The mean alkaline phosphatase specific activities and total protein content for the various hypophysectomized embryos (groups 2-11) were recalculated and compared separately for embryos with a patent or non-patent esophagus. These data appear in Table 15. To overcome small sample sizes, the groups were pooled according to treatments which stimulated repair of duodenal development (groups 3, 4 and 6-8) and treatments which did not stimulate repair (groups 2, 5 and 9). Groups 10 and 11 were omitted since they had values intermediate between the normal (group 1) range and the hypophysectomized (group 2) range. Only the 19.5 and 20.5-day classes were compared statistically.

Duodenal alkaline phosphatase specific activity is not affected appreciably by esophageal non-patency until 19.5 days in unstimulating treatments. At this time, the phosphatase value for the embryos with a patent esophagus is significantly higher than that for embryos with a non-patent esophagus (Table 15). This difference persists at 20.5 days. In the stimulating treatment groups alkaline phosphatase activities appear to be elevated in embryos with a patent esophagus from 18.5 days on, but the difference does not become significant even at the 0.10 level until 20.5 days.

TABLE 15 EFFECT OF ESOPHAGEAL PATENCY ON ALKALINE PHOSPHATASE SPECIFIC ACTIVITY $\frac{(\mu\text{g/hr})}{(\text{mg})}$ AND TOTAL PROTEIN (mg) IN THE DUODENUM

Parameter	Treatment	Days of Incubation				
		15.5	16.5	17.5	18.5	19.5 20.5
Alkaline phosphatase (Activity/Protein)	Unstimulated*	P** 248(5)++	289(10)	318(14)	313(13)	527(14) 541(20)
	NP	371(9)	365(4)	318(17)	306(6)	328(12) 378(9)
	Stimulated†	P -	-	718(13)	2115(17) ¹	2282(17) ¹ 5604(41) ²
	NP	-	-	725(4)	1689(5)	1541(9) ¹ 3680(10) ²
Total Protein	Unstimulated	P 0.6(5)	1.1(10)	1.7(14)	2.0(13)	2.6(14) 3.0(20)
	NP	0.5(9)	1.3(4)	1.9(17)	2.0(6)	2.2(12) 2.4(9)
	Stimulated	P -	-	2.5(13)	3.4(17)	4.1(17) 4.7(41) ¹
	NP	-	-	2.2(4)	2.5(5)	3.2(9) 4.3(10) ¹

* Includes group 2 at 15.5 and 16.5 days, groups 2 and 5 at 17.5-19.5 days and groups 2, 5 and 9 at 20.5 days.

† Includes groups 3 and 4 at 17.5-19.5 days and groups 3, 4, 6, 7 and 8 at 20.5 days.

** P = patent; NP = non-patent

++ Mean and # of samples (in parentheses).

¹ Means for the 19.5 and 20.5 day class only, with the same superscript were not significantly different as compared by 'Student's' t-test, P = 0.05.

² These means only became significantly different at P = 0.10.

A similar pattern is evident from the total protein values for the unstimulating treatments. Unstimulated embryos with a patent esophagus have significantly higher duodenal total protein at 19.5 and 20.5 days than their counterparts with a non-patent esophagus. The pattern with stimulating treatments is disturbed, however. Embryos with a patent esophagus had significantly elevated duodenal total protein by 19.5 days but the difference disappeared at 20.5 days.

Also the morphology of the duodenum may be slightly different with esophageal patency (Figures 51, Plate VI and 52, Plate VII). The height of the epithelium and thickness of the brush border are slightly increased in embryos with a patent esophagus (Figure 51, Plate VI) compared to those from the same group but with a non-patent esophagus (Figure 52, Plate VII).

Generally, embryos with a patent esophagus tended to have increased levels of duodenal development in unstimulating treatment groups during the last two days of incubation but the duodena in stimulating treatment groups appeared to be less affected.

Characteristics of the Thyroid Glands

The fixed weights of thyroid glands from each group at 20.5 days are listed in Table 16. Groups 6 and 10 had fixed weights of thyroid glands within the normal range. Groups 3, 4, 7, 8 and 11 were subnormal

TABLE 16 FIXED WEIGHTS (mg) OF THYROID
GLANDS FROM ALL GROUPS AT 20.5
DAYS

Group	Weight
1	$2.8 \pm 0.2^*(21)^+(a)^x$
2	0.9 ± 0.1 (8) (c)
3	2.1 ± 0.2 (8) (b)
4	2.0 ± 0.1 (9) (b)
5	0.6 ± 0.1 (8) (c)
6	2.3 ± 0.3 (6) (a), (b)
7	1.8 ± 0.2 (8) (b)
8	1.9 ± 0.2 (6) (b)
9	1.1 ± 0.2 (5) (c)
10	2.2 ± 0.3 (4) (a), (b)
11	2.0 ± 0.3 (4) (b)

* Mean $\pm$ S.E.

+ # of samples

x Means followed by same letter (in parentheses)
are not significantly different.

but not significantly lower than groups 6 and 10. Also, these groups had significantly heavier thyroid glands than did group 2. Only groups 5 and 9 had thyroid gland weights not different from group 2.

The histology of representative thyroid glands from each group at 20.5 days is in Figures 63-66, Plate VIII and Figures 67-73, Plate IX. The follicles in thyroid glands from hypophysectomized embryos (Figure 64, Plate VIII) were much smaller than normal (Figure 63, Plate VIII), more sparsely distributed and frequently devoid of stainable colloid. Many of the cells were in cords among the follicles.

Thyroid glands from embryos in groups 3, 4, 6-8, 10 and 11 (Figures 65 and 66, Plate VIII and Figures 69-73, Plate IX) were within the normal range of variation as regards follicle size and amount of colloid.

Thyroid glands in groups 5 and 9 (Figures 67 and 68, Plate IX) had small follicles of the same size range as those in the thyroid glands of group 2 embryos (Figure 64, Plate VIII). In general, the thyroid glands of these two groups did not appear to be stimulated, however, group 5 embryos appeared to have relatively fewer cords of cells among the follicles than in group 2. Also, the follicular cells in group 9 embryos appeared to be hypertrophied as compared to group 2.

In all groups in which cephalic region pars distalis tissue was present in the graft (3, 4, 6-8, 10 and 11) thyroid gland fixed weights and histology were substantially improved over those of group 2 embryos. Groups 5 and 9 did not exhibit repair of the thyroid deficiency characteristic of group 2 embryos.

Histology of Normal and Grafted Partes Distales

The histology of representative cephalic, middle and caudal regions of the partes distales from group 1 at 20.5 days is illustrated in Figures 74, 76 and 78, respectively (Plate X). No obvious regional differences were apparent in staining characteristics with acid fuchsin and toluidine blue, however, granular basophils and darkly stained acidophils were easily identified (Figures 74 and 76, Plate X). Differential cell counts were not made.

The histology of representative pars distalis graft regions from 20.5 day-old embryos is illustrated in Figures 75, 77 and 79-85, Plate X. All graft cells appeared normal.

DISCUSSION

The interpretations derived from hypophysectomy and extirpation experiments usually are supplemented by those from replacement therapy experiments which involve administration of homografts of the missing gland, or extracts of the missing gland or purified hormones. Probably the grafting method most closely approaches conditions in the intact embryo as it usually eliminates problems such as effects due to abnormal dosage and/or improper route of administration. Also, in the absence of readily available preparations of purified chick pars distalis hormones, pars distalis homografts are perhaps the best source of isospecific hormones. The regional grafting technique which apparently achieves partial isolation of pars distalis hormone activities (Brasch and Betz, 1971) was thus considered the most advantageous for this study.

Thyroid status was selected as a control for indicating graft function since it has been well-documented and exhibits the most striking histological differences between the stimulated and unstimulated conditions (Betz, 1967; 1971; Brasch and Betz, 1971).

As pointed out in the introduction, duodenal alkaline phosphatase activity (although its relationship to duodenal function is poorly understood) has

been used extensively as a remarkably sensitive indicator of duodenal maturation (Moog and Richardson, 1955; Hinni and Watterson, 1963; Bellware and Betz, 1970). This is probably justified as long as additional parameters are also examined as was done here.

The individual variation in duodenal alkaline phosphatase activity produced complications in statistical analyses of the results. From 17.5 days on, the mean values for phosphatase activities for various age classes in stimulating treatment groups had very high standard errors. In extreme cases, means 3 to 4 times greater than a given mean value could not be shown significantly different by the Duncan's new multiple range test. Furthermore, means which were significantly different when treated in the context of a small series of means, e.g. comparison of groups 1-5 at 20.5 days, could not be shown significantly different when treated in the context of a larger series of means, e.g. comparison of groups 1-11 at 20.5 days. Such situations made it advisable to interpret the statistics with caution and indicated that a statistical test which compensates for disproportionate amounts of variance (from group to group) may have been preferable for analysis of the data in this study.

The variability of the alkaline phosphatase data may have originated from a number of sources. It

seems unlikely that preparation of homogenates for the assay procedure introduced much variability, however, since total protein data measured from the same homogenates did not exhibit a proportionate variability. The most obvious source of variability must be introduced by factors affecting the physiological age (developmental level) of the embryos. These would include handling of eggs during shipment, duration of preincubation storage and possible seasonal differences in developmental rates. In addition, differences in incubation time could be introduced when eggs were removed from the incubator for experimental treatment, e.g. hypophysectomy, grafting, or autopsy. Apparently small differences in physiological age introduced in any of the above ways would add to the variability in the data, especially during the rapid upsurge of alkaline phosphatase activity. In any case, considerable inherent variability is apparently a characteristic of the phosphatase system in the duodenum of the chick embryo even under standard conditions (Moog and Richardson, 1955).

The differences observed between groups in external features of the embryos indicated that the cephalic region of the pars distalis is essential for normal skin and feather development and for normal retraction and vascular reduction of the yolk sac.

Hypophysectomy apparently arrested feather development and skin keratinization but these deficiencies were repaired by cephalic region and middle region (containing cephalic region tissue) homografts. These observations concur with those of Betz (Betz, 1967; 1968; Bellware and Betz, 1970), but show some discrepancy with those of Brasch and Betz, (1971). These are subjective observations, however, and a more critical analysis is warranted. Goeringer (1968) showed that hypophysectomized chick embryos had significantly delayed epidermal differentiation and anomalous feather growth and development. Johnson (1968) reported deficient down feather barbule cell development in either decapitated or thiourea-treated embryos. Thyroxine administration produced partial correction of this deficiency. In addition, thiourea-injected embryos had significantly delayed morphogenesis and keratinization of scales apparently due to deficient keratin synthesis but thyroxine repaired these parameters to normal or precocious levels (Lawrenz and Johnson, 1970). The stimulation of epidermal development by cephalic region pars distalis tissue, observed in the present study, was likely a result of TSH stimulation of the thyroid glands to produce normal titers of thyroxine. This is supported by the repair of thyroid histology and fixed weights seen in the groups with cephalic region

tissue in the grafts, and also by previous findings (Betz, 1971; Brasch and Betz, 1971).

Moog and Richardson (1955) found an acceleration of yolk sac retraction in chick embryos by doses of adrenocorticoid which induced precocious duodenal development. The repair of yolk sac retraction observed here might therefore be due to stimulation by the cephalic region of the pars distalis of normal corticoid titers from the adrenal cortex. This is compatible with previous reports indicating that ACTH activity originates in the cephalic region (See Brasch and Betz, 1971).

Both cephalic and caudal regions of the pars distalis improved survival rates of hypophysectomized embryos. The results found here have no serious discrepancies with those of Betz (1967), although his results indicated a higher mortality among hypophysectomized embryos and a lower mortality for pars distalis-grafted embryos on day 20. Brasch and Betz (1971), however, reported little improvement of survival in grafted embryos. The mortality rates recorded here should be interpreted with caution since the method of determination may have introduced certain biases. Mortality was recorded at the time of autopsy without prior checks. Embryos were placed in a group after completion of any experimental manipulations prior to autopsy, e.g. normal embryos were classified

as group 1 from the start of incubation; hypophysectomized embryos were classified as group 2 from the termination of the partial decapitation operation; and grafted embryos were classified in their respective groups after the grafting operation. Thus, embryos dying between 0 and 1.5 days would not contribute to group 2 mortality rates and embryos dying between 1.5 and 10.5 days would not contribute to mortality rates of grafted groups. This would tend to lower graft group mortality rates perhaps considerably and to lower group 2 mortality rates to a lesser degree. Furthermore, mortality in grafted groups might tend to be increased by the additional experimental manipulation of these embryos. In addition, except for 24 hours after grafting, the condition of grafts was not checked until autopsy. However, if embryos alive in each group one day after grafting (to eliminate traumatic effects of grafting procedure) were taken as 100% of the potential survivors for each group and daily mortality checks were made, a more valid comparison of mortality rates could be obtained.

Body growth was significantly reduced by hypophysectomy as shown previously (Betz, 1967; 1968; 1971; Bellware and Betz, 1970; Brasch and Betz, 1971). Body weight and third toe length in 20.5 day-old group 2 embryos was higher in this study than those reported by Betz (1967) and by Brasch and Betz (1971). Third

toe lengths reported here also are higher than those found by Bellware and Betz (1970). There is no apparent reason for these discrepancies except possible strain differences. The cephalic region of the pars distalis supported repair of third toe lengths but no significant improvement of body weights compared to hypophysectomized embryos at 20.5 days. Caudal region pars distalis grafts failed to repair either parameter. These results are in general agreement with those of Brasch and Betz (1971) except that they found significant repair of third toe length with caudal region grafts as well. Again, the explanation for the apparent discrepancy is not clear.

However, the body growth results here are consistent with those of King (1969), who reported declines in body weights and shank-middle toe lengths in hypophysectomized young cockerels. The latter parameter was more severely affected in King's study as it was in this study. Moreover, the reduced body weights observed by King were partly due to a lower food intake by hypophysectomized cockerels and force feeding which restored these body weights still failed to restore bone growth to normal. Thyroxine restored this deficient bone growth. In addition, Mehall (1970) recently reported reduced growth in long bones (but not dermal bones) of hypophysectomized chick embryos

but this was restored by pars distalis grafts. He suggested STH and/or TSH were probably responsible for this correction. Thus in the light of the above studies, repair of third toe lengths by cephalic region pars distalis grafts is probably due to TSH stimulation of normal thyroid hormone titers. The absence of repair by caudal region grafts suggests that STH alone is not capable of restoring growth of long bones.

Duodenal development (in terms of fresh weight, total protein, alkaline phosphatase activity and histology) was arrested or severely retarded by hypophysectomy. Fresh weights increase 3.5-fold, total protein- 5-fold and specific activity of alkaline phosphatase- nearly 20-fold from 15.5 to 20.5 days in normal embryos. The histological parameters exhibited similar but less striking changes during the same period. In hypophysectomized embryos the values for these parameters were invariably lower (though not always significantly so) and the increases from 15.5 to 20.5 days were usually smaller: fresh weight- approximately 2.5-fold, total protein- 5-fold and specific activity of alkaline phosphatase- less than 2-fold. Duodenal morphogenesis was arrested at a level comparable to that for 17.5-day normal embryos. These results are generally in good agreement with Hinni and Watterson (1963) and Bellware and Betz (1970).

Grafts containing cephalic region pars distalis tissue repaired the defects of duodenal ontogeny to normal or nearly normal levels by 20.5 days but caudal region grafts without exception failed to improve upon the hypophysectomized level of development.

In connection with the work of Moog and her colleagues (reviewed in the introduction) and with the findings of Brasch and Betz (1971), the evidence presented here strongly indicates that the cephalic region of the pars distalis achieved correction of the defective duodenal development in hypophysectomized embryos through secretion of ACTH and TSH which stimulated the thyroid glands and adrenal cortices to produce normal titers of their respective hormones. If STH is confined to the caudal region (Enemar, 1967; see Brasch and Betz, 1971) then it does not appear to be essential for restoration of normal duodenal development nor is it capable (in isolation from ACTH and TSH) of causing normal duodenal development. Thus far, no report has implicated FSH, MSH or LH to be operative in this system. There is some evidence, however, that gonadal steroids in large doses are capable of stimulating precocious induction of alkaline phosphatase activity in the duodenum of the nursing mouse (Moog and Thomas, 1955) but in the present study, the ineffectiveness of caudal region grafts indicates that LH (acting via the

gonads) is probably not important for duodenal development in the chick embryo. The question of whether or not prolactin may be involved in duodenal development in chick embryos remains unresolved. Bates et al. (1962) found that mammalian prolactin or growth hormone (STH) partially restored intestinal and crop sac weights of hypophysectomized juvenile pigeons. In the presence of thyroxine and an adrenocorticoid (which had no effect alone or together) the prolactin and STH effects were heightened. The authors suggested ACTH and TSH have a permissive action in conjunction with either prolactin or STH. Whether or not prolactin contributed to the success of cephalic region grafts in restoring normal duodenal development cannot be ascertained from present evidence. In addition, since mammalian STH has been shown in vitro to support increased alkaline phosphatase levels in the duodenum of normal and hypophysectomized chick embryos (Struthers and Betz, unpublished data), the possibility that some STH activity may be present in the cephalic region should not be ignored.

In concurrence with Bellware and Betz (1970), duodenal development in hypophysectomized embryos was restored to normal or nearly normal levels by entire pars distalis grafts. The entire pars distalis, however, did not prove more effective than a single cephalic

region graft, a fact which emphasizes the notion that the cephalic region must be capable of supplying all of the endocrine factors essential for normal duodenal development.

Regional double grafts had effects no different from the respective single region grafts as regards duodenal development. Betz (1967) also found multiple grafts of entire partes distales no more effective than single grafts as judged by repair of various parameters in hypophysectomized embryos. Evidently, the secretory levels of these double grafts are regulated at a level not in excess of that for single region grafts. Possibly, a direct feedback system not involving the hypothalamus is in operation here. The results of Betz (1967) are consistent with this hypothesis. Also, subcutaneous hypophyseal homografts restored to normal the iodine-binding capacity of the thyroid glands in decapitated chick embryos (Mess and Straznicky, 1964). Furthermore, thiouracil treatment of decapitated embryos with transplanted pituitaries resulted in goitre (Mess and Straznicky, 1969) indicating thyroid-pituitary feedback in the absence of the hypothalamus. Bohus et al. (1970) reported that metyrapone administration induced significant adrenal ascorbic acid depletion in both intact embryos and decapitated embryos bearing a subcutaneous pituitary graft. They suggested the embryonic pituitary was "highly auto-

monic" in respect of adrenocorticotrophic function. Furthermore, Frankel (1970) points out in his review that avian adrenal glands themselves apparently possess considerable autonomy. Betz (1971) has reviewed the evidence for regulation of the embryonic pars distalis without hypothalamic control. However, hypothalamic regulation of feedback evidently becomes established soon after hatching (Bohus et al., 1970) since hypothalamic feedback systems appear to be present in the chicken some time after hatching (Kanematsu and Mikami, 1969).

The failure of double grafts to exceed the effects of single region grafts might alternatively be explained by the production, by single cephalic grafts, of hormone levels sufficient to elicit maximum response. Thus excessive levels of secretion may not be detectable in terms of improved duodenal differentiation. The fact that Moog and Richardson (1955) were unable to stimulate the chick embryo duodenum to levels beyond the 19-day maximum with increasing doses of corticoid may be consistent with this notion.

The grafts of cephalic + caudal regions (group 10) and cephalic + middle + caudal regions (group 11) were consistently the least effective of those graft types which contained cephalic region pars distalis tissue. Possibly the presence of a caudal region exerted some

inhibitory effect on the cephalic region, but Brasch and Betz (1971) were not of this opinion, as they reported apparently normal function of isolated cephalic and caudal region grafts indicating that the regionalization of functions of the in situ pars distalis apparently does not depend upon regional interactions. Furthermore, the success of entire pars distalis grafts in repairing duodenal development in hypophysectomized embryos also militates against the idea of an inhibitory effect by the caudal region. This point must be clarified by further investigation.

On the other hand, the lower efficiency of grafts in groups 10 and 11 may be due to the grafting technique. When the pars distalis was segmented for multiple grafts the respective regions were crushed together and placed as a single graft (containing two or more regions) on the chorioallantois. One might, a priori, expect a greater degree of necrosis in grafts of larger mass, viz. multiple grafts, than in grafts of smaller mass, viz. single region grafts. The fact that double cephalic region grafts were apparently less effective (but not significantly so) in stimulating certain duodenal parameters than were single cephalic region grafts, is compatible with this explanation. Entire pars distalis grafts, although of large mass, were not segmented prior to crushing and so might be

expected to have a higher proportion of intact cells. In any case, further investigation is necessary to resolve this problem.

In the absence of pars distalis stimulation, embryos with a patent esophagus had significantly improved duodenal development at 19.5 and 20.5 days compared to their counterparts having a non-patent esophagus. According to Rupe and Farmer (1955), embryos from 10 to 16 days took up amino acids from the albumen almost exclusively. Betz (1968) showed that swallowed albumen was associated with increased body growth, but only in the presence of pars distalis stimulation. He did not examine duodenal parameters, however. The evidence here indicates that in the absence of pars distalis stimulation, the dietary albumen swallowed by embryos with a patent esophagus can significantly increase the rate of duodenal development compared to embryos with a non-patent esophagus. However, differences in total protein content between the patent and non-patent groups may in part be due to non-specific adsorption of protein to the surface of the microvilli (Taylor, 1970) in the duodena of the patent group. Presumably the mucopolysaccharides of the glycocalyx are important in this respect (Martínez-Palomo, 1970). Nevertheless, the fact that alkaline phosphatase activity was also elevated in the embryos with a patent esophagus probably cannot be accounted

for by non-specific adsorption of protein, since albumen does not contain alkaline phosphatase detectable by the method used here (Bellware, 1969). In fact, one might expect an apparent lowering of specific activity in the presence of non-specific protein adsorption. It is doubtful that the lower phosphatase activities associated with the non-patent group were caused by starvation since Moog (1950) reported elevation of phosphatase activity in the duodenum of newly-hatched chicks which were fasted.

The effect of esophageal patency was not as great in the embryos which received stimulating treatments. This may simply be due to the insensitivity of the duodenal parameters to other stimuli since they may already be near maximum stimulation.

This apparent effect of dietary albumen may represent an alteration of duodenal alkaline phosphatase activity not mediated by hormones. In this connection, it is interesting to note that Cook and Moog (1970) reported a reduction of duodenal alkaline phosphatase activity in the mouse under the influence of biliary stasis. This may also be a nonhormonal effect. However, unlike the albumen effect, biliary stasis did not alter protein content in the duodenum. In any case, the effect of esophageal patency on duodenal development deserves further examination.

Thus, during the final three days of incubation, the chick embryo duodenum undergoes a period of intensive differentiation which is apparently dependent upon the thyro-pars distalis and adrenocortico-pars distalis axes with the possible additional support of other pars distalis hormones. Most of the changes occurring in the duodenum at this time have been related to a preparation for increased absorptive function or to a termination of certain absorptive properties of the embryonic duodenum.

The mucosa acquires a remarkably enlarged surface area including an increase in the thickness and extent of the brush border. The development of a mucopolysaccharide glycocalyx or surface coat which is evidently an integral part of the apical plasma membrane of mucosal cells (Ito, 1969) occurs at the same time. The functional significance of the glycocalyx is still a matter of some dispute (Martínez-Palomo, 1970; Taylor, 1970). It has been variously ascribed the functions of a filter regulating access of substrates to the enzymes of the brush border, or a simple mechanical support which protects and maintains the integrity of the mucosal surface, or a surface for adsorption of substrates and/or extracellular enzymes thus promoting surface digestion.

The PAS-positive granules which disappeared from the mucosal cells during the terminal stages of develop-

ment were apparently glycogen (Moog and Thomas, 1957; Hinni and Watterson, 1963). This may serve as an energy source for differentiation (Moog and Richardson, 1955). The apparent failure of amylase digestion to verify this identification in the present study was disappointing. However, the fact that the sections of duodenum were heat-dried on the slides may have resulted in sealing of the plastic over the surface of the section rendering the substrate inaccessible (G. Setterfield, personal communication). A further attempt at amylase extraction using air-dried sections may solve this problem.

The immense increase in alkaline phosphatase activity also occurs concomitantly with brush border development (Moog, 1965). The enzyme is localized in the region of the brush border and although its specific role in duodenal function remains unknown, the correlation of alkaline phosphatase activity changes with those of other developmental parameters has been well-documented (Moog, 1958; 1959b; 1965; see also introduction). (Histochemical localization of alkaline phosphatase was not attempted in the present study as the fixation and embedding procedures used here were not amenable for enzyme histochemical studies.)

There is increasing evidence that the appearance of transport functions in the duodenum coincides with

the increase in alkaline phosphatase activity (Pratt and Turner, 1971; see also introduction). Indeed, alkaline phosphatase changes often appear to anticipate imminent changes in diet and Moog (1965) considers alterations in the activity of this enzyme to be an integral part of the functional differentiation of the duodenum.

Loss of the ability of the duodenum to absorb proteins in the mouse coincides with the first appearance of alkaline phosphatase detectable with electron microscopic histochemistry (Hugon, 1970). In this connection, it is interesting to note that Clarke and Hardy (1969) found that the disappearance of phagocytic vacuoles in the mucosa occurred concomitantly with the loss of ability to absorb macromolecules in the neonatal rat. Unfortunately, the biological significance of the disappearance of perinuclear and other vacuoles observed here is not as apparent.

The results of the present study have suggested a number of problems for investigation:

1. Demonstration of alkaline phosphatase localization in the region of the brush border by means of both light and electron microscopic histochemistry of chick embryo duodena.
2. Analysis of ultrastructural changes associated with duodenal functional differentiation by means of electron microscopy of groups in the

present experimental design.

3. Examination of the effect of esophageal patency on duodenal development employing larger sample sizes and additional developmental parameters. Suitable controls, e.g. upper beakless embryos and embryos without a supply of albumen should be included in such a study and perhaps confining treatment to a specific graft type, e.g. whole partes distales, may reduce the variation.
4. Extraction and purification of the regional trophic hormone activities from the pars distalis and from regional grafts with a view to verifying the secretions of the gland and confirming the roles of ACTH and TSH in duodenal development. Investigation of the involvement of additional pars distalis hormones, especially STH and/or prolactin, in duodenal development.
5. A study of the development of a variety of transport systems, e.g. glucose transport, amino acid transport, in the context of the present experimental design. This would ascertain whether or not development of duodenal function is closely linked with the parameters explored in the present study.
6. Examination of regulation in pars distalis grafts (in the absence of the hypothalamus) em-

ploying inhibitors of end organ function or of specific pars distalis hormones.

7. A critical cytological study, using both light and electron microscopy, of (a) normal partes distales, (b) entire pars distalis grafts and (c) segmented pars distalis grafts (group 11), in correlation with bioassay of the secretory activity of each, may indicate possible interactions between regions. Both in vivo and in vitro methods could be used.

SUMMARY

The effects of pars distalis hormones on duodenal development in the chick embryo were analyzed using the techniques of hypophysectomy and replacement therapy with grafts of the cephalic and caudal regions of the pars distalis. A variety of parameters of body growth and of duodenal development were examined:

1. The external features of the embryos exposed to different treatments indicated that skin and feather development, (deficient in hypophysectomized embryos) was repaired by the hormone activities of the cephalic region but not by those of the caudal region.
2. Cephalic region grafts repaired growth of the third toe but not the body weights of hypophysectomized embryos. Caudal region grafts had no effect on either parameter.
3. Several developmental parameters in the duodenum were repaired to normal or at least greatly improved over hypophysectomized levels by cephalic region grafts. Caudal region grafts had no effect.
4. Entire pars distalis grafts or 2 cephalic region grafts were no more effective than a single cephalic region as judged by their ability to

support duodenal development. Two caudal region grafts were as ineffective as a single caudal region graft in this respect.

5. Grafts of a cephalic + a caudal region and grafts of segmented partes distales were consistently less effective than whole partes distales or single cephalic region grafts in supporting normal duodenal development.
6. In the absence of pars distalis stimulation, embryos with a patent esophagus had duodenal development significantly advanced (as judged by total protein content and alkaline phosphatase activity) over their counterparts having a non-patent esophagus. Under conditions of pars distalis stimulation, this effect was depressed.
7. The results indicated that TSH and ACTH from the cephalic region of the pars distalis are important for normal duodenal development. Whether or not additional pars distalis hormones are involved is still not clear.
8. Apparently grafts were secreting regulated levels of hormones so that there was no increase in stimulation by doubling the amount of tissue grafted. Alternatively, the secretory levels of single region grafts were sufficient for

maximal stimulation so that excessive secretory levels in double grafts might not be detected.

9. The differences in effect between cephalic and caudal region grafts reflect qualitative rather than quantitative differences in the hormone activities of the two regions.
10. Dietary albumen appeared capable of augmenting development in duodena not already maximally stimulated.

PLATE II

Histology of glutaraldehyde-fixed, glycol methacrylate-embedded duodena sectioned at 2μ and stained with acid fuchsin and toluidine blue. Scale line (Figure 19) represents 23.6μ (low power) and 5.9μ (high power inserts) in Figures 19-24.

Figure 19. Duodenum from 15.5 day-old group 1 embryo.
Note strong basophilic cytoplasm of nearly cuboidal mucosal cells. No detectable brush border. Note also erythrocyte aggregation (arrow) in stroma.

Figure 20. Duodenum from 15.5 day-old group 2 embryo.
No trace of brush border is present.

Figure 21. Duodenum from 16.5 day-old group 1 embryo.
Compare with Figure 19.

Figure 22. Duodenum from 16.5 day-old group 2 embryo.
Note perinuclear vacuoles (arrow in insert).
Similar to Figures 19-21.

Figure 23. Duodenum from 17.5 day-old group 1 embryo.
Note cells are low columnar in profile (insert) with first suggestion of brush border (arrow) at their apices.

Figure 24. Duodenum from 17.5 group 2 embryo. Similar to Figure 23 but without traces of brush border.

PLATE III

Histology of duodena (as in Plate II). Scale line (Figure 25) represents 23.6μ (low power) and 5.9μ (high power inserts) in Figures 25-30.

Figure 25. Duodenum from 17.5 day-old group 3 embryo.
Compare with Figure 23.

Figure 26. Duodenum from 17.5 day-old group 4 embryo.
Compare with Figure 23. Note size of perinuclear vacuole (arrow insert).

Figure 27. Duodenum from 17.5 day-old group 5 embryo.
Compare with Figure 24.

Figure 28. Duodenum from 18.5 day-old group 1 embryo.
Cells are distinctly columnar and apical cytoplasm has a granular structure (arrow in insert). Brush border present but indistinct. Vacuoles less frequent.

Figure 29. Duodenum from 18.5 day-old group 2 embryo.
Similar to 17.5 day normal condition (Figure 23). Contrast with Figure 28.

Figure 30. Duodenum from 18.5 day-old group 3 embryo.
Compare with Figure 28 and 29. Brush border (arrow in insert) more distinct than in Figure 28.

PLATE IV

Histology of duodena (as in Plate II). Scale line (Figure 31) represents 23.6μ (low power) and 5.9μ (high power inserts) in Figures 31-36.

Figure 31. Duodenum from 18.5 day-old group 4 embryo.
Compare with Figure 28.

Figure 32. Duodenum from 18.5 day-old group 5 embryo.
Note absence of detectable brush border.
Mucosal cells are cuboidal to low columnar.
Compare with Figures 28 and 29.

Figure 33. Duodenum from 19.5 day-old group 1 embryo.
Note columnar cells (insert) and reduction in basophilia compared to earlier stages (Figures 23 and 28).

Figure 34. Duodenum from 19.5 day-old group 2 embryo.
Note intense basophilia and frequent vacuoles in mucosal cell cytoplasm. Cells low columnar.

Figure 35. Duodenum from 19.5 day-old group 3 embryo.
Similar to 19.5 day normal (Figure 33).

Figure 36. Duodenum from 19.5 day-old group 4 embryo.
Compare with Figure 33.

PLATE V

Histology of duodena (as in Plate II). Scale line (Figure 37) represents 23.6μ (low power) and 5.9μ (high power inserts) in Figures 37-42.

Figure 37. Duodenum from 19.5 day-old group 5 embryo.

Mucosal cells low columnar with frequent large vacuoles (arrow in insert) and intense basophilia in cytoplasm. No brush border. Compare with Figures 33 and 34.

Figure 38. Duodenum from 20.5 day-old group 1 embryo.

Note absence of basophilia and granular appearance of mucosal cell cytoplasm. Cells are tall columnar with distinct brush border (arrow in insert).

Figure 39. Duodenum from 20.5 day-old group 1 embryo.

This shows the lower end of the normal range of variation at 20.5 days. Mucosal cells are not as tall as in Figure 38 and cytoplasm is more basophilic.

Figure 40. Duodenum from 20.5 day-old group 2 embryo.

The level of differentiation is not improved over the 17.5 day normal condition (Figure 23).

Figure 41. Duodenum from 20.5 day-old group 3 embryo.

Very tall mucosal cells with distinct granular structure (arrow in insert) in apical cytoplasm and a well-defined brush border.

Figure 42. Duodenum from 20.5 day-old group 4 embryo.
Compare with Figures 38 and 39.

PLATE VI

Histology of duodena. Figures 43, 50 and 51 as in Plate II. Figures 44-49 stained with the PAS technique and fast green. Scale line (Figure 43) represents $23.6\ \mu$ (low power) and $5.9\ \mu$ (high power inserts) in Figures 43, 50 and 51. Scale line (Figure 44) represents $5.9\ \mu$ in Figures 44-49.

Figure 43. Duodenum from 20.5 day-old group 5 embryo. Similar to 20.5 day group 2 (Compare with Figures 23 and 40).

Figure 44. Duodenum from 20.5 day-old group 1 embryo. Note PAS-positive border (arrow).

Figure 45. Duodenum from 20.5 day-old group 2 embryo. PAS-positive border is absent but the mucosal cells contain PAS-positive granules (arrow).

Figure 46. Same as Figure 45 but pretreated with amylase extraction. Cytoplasmic PAS-positive granules are finer and slightly less abundant.

Figure 47. Duodenum from 20.5 day-old group 3 embryo. Compare with Figure 44. Border is not as strongly PAS-positive. Note goblet cell (arrow).

Figure 48. Duodenum from 20.5 day-old group 4 embryo. Compare with Figure 44.

Figure 49. Duodenum from 20.5 day-old group 5 embryo.

Compare with Figure 45.

Figure 50. Duodenum from 20.5 day-old group 6 embryo.

Compare with Figure 39.

Figure 51. Duodenum from 20.5 day-old group 7 embryo.

Compare with Figures 38 and 41.

PLATE VII

Histology of duodena. Figures 52-56 as in Plate II. Figures 57 and 58 stained with the PAS technique and fast green. Scale line (Figure 52) represents 23.6μ (low power) and 5.9μ (high power inserts) in Figures 52-56. Scale line (Figure 57) represents 5.9μ in Figures 57 and 58.

Figure 52. Duodenum from 20.5 day-old group 7 embryo with a non-patent esophagus. Compare with Figure 51.

Figure 53. Duodenum from 20.5 day-old group 8 embryo. Compare with Figures 38 and 42.

Figure 54. Duodenum from 20.5 day-old group 9 embryo. Compare with Figures 40 and 43.

Figure 55. Duodenum from 20.5 day-old group 10 embryo. Compare with Figures 39 and 40. Mucosal cells do not appear to be better differentiated than the hypophysectomized condition at 20.5 days (Figure 40).

Figure 56. Duodenum from 20.5 day-old group 11 embryo. Compare with Figure 39.

Figure 57. Duodenum from 20.5 day-old group 6 embryo. Note PAS-positive granules in basal cytoplasm (arrow). Compare with Figure 44.

Figure 58. Duodenum from 20.5 day-old group 7 embryo. Compare with Figure 47.

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PLATE VIII

Histology of duodena. Figures 59-62 stained with the PAS technique and fast green. Histology of glutaraldehyde-fixed, glycol methacrylate-embedded thyroid glands (from 20.5 day-old embryos) (Figures 63-66) sectioned at 2μ and stained with acid fuchsin and toluidine blue. Scale line (Figure 59) represents 5.9μ in Figures 59-62. Scale line (Figure 63) represents 14.8μ in Figures 63-66.

Figure 59. Duodenum from 20.5 day-old group 8 embryo.
Compare with Figure 48.

Figure 60. Duodenum from 20.5 day-old group 9 embryo.
Compare with Figure 49.

Figure 61. Duodenum from 20.5 day-old group 10 embryo.
Note PAS-positive granules in basal cytoplasm of mucosal cells. Compare with Figure 44.

Figure 62. Duodenum from 20.5 day-old group 11 embryo.
Compare with Figures 44 and 57.

Figure 63. Thyroid gland from group 1 embryo. Note size of colloid-filled follicles.

Figure 64. Thyroid gland from group 2 embryo. Follicles are much smaller and frequently contain no stainable colloid. Compare with Figure 63.

Figure 65. Thyroid gland from group 3 embryo. Compare with Figure 63.

Figure 66. Thyroid gland from group 4 embryo. Follicle cells have more abundant vacuoles (arrow) than in normal thyroid gland (Figure 63).

PLATE IX

Thyroid gland histology (as in Plate VIII). Scale line (Figure 67) represents 14.8μ in Figures 67-73.

Figure 67. Thyroid gland from group 5 embryo. Compare with Figure 64.

Figure 68. Thyroid gland from group 9 embryo. The cells appear hypertrophied. Compare with Figures 64 and 67.

Figure 69. Thyroid gland from group 6 embryo. Compare with Figure 63.

Figure 70. Thyroid gland from group 7 embryo. Compare with Figure 65.

Figure 71. Thyroid gland from group 8 embryo. Compare with Figure 66.

Figure 72. Thyroid gland from group 10 embryo. Compare with Figure 64.

Figure 73. Thyroid gland from group 11 embryo. Compare with Figures 63 and 69.

PLATE X

Histology of glutaraldehyde-fixed, glycol methacrylate-embedded pars distalis regions and pars distalis region grafts (from 20.5 day-old embryos) sectioned at 2μ and stained with acid fuchsin and toluidine blue. Scale line (Figure 74) represents 5.9μ in Figures 74-85.

- Figure 74. Cephalic region from normal (group 1) pars distalis. Note darkly stained acidophils (large arrow) and basophils (small arrow) with fine granules.
- Figure 75. Graft of a single cephalic region of the pars distalis (group 3). Compare with Figure 74.
- Figure 76. Middle region of normal (group 1) pars distalis. Note basophil (arrow).
- Figure 77. Graft of a single middle region of the pars distalis (group 4). Compare with Figure 76.
- Figure 78. Caudal region of normal (group 1) pars distalis.
- Figure 79. Graft of a single caudal region of the pars distalis (group 5). Compare with Figure 78.
- Figure 80. Graft of entire pars distalis (group 6). Compare with Figures 74, 76 and 78.
- Figure 81. Graft of two cephalic regions of the pars distalis (group 7). Compare with Figures 74 and 75.

- Figure 82. Graft of two middle regions of the pars distalis (group 8). Compare with Figures 76 and 77.
- Figure 83. Graft of two caudal regions of the pars distalis (group 9). Compare with Figures 78 and 79.
- Figure 84. Graft of one cephalic and one caudal regions of the pars distalis (group 10). Compare with Figures 74 and 78.
- Figure 85. Graft of one cephalic, one middle and one caudal regions of the pars distalis (group 11). Compare with Figures 74, 76, 78 and 80.

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APPENDIX I

Sample calculation for assay of total protein:

$$\text{Amount of protein (mg)} = \frac{\text{O.D.}_{\text{obs.}}}{\text{O.D.}_{\text{st.}}} \times \frac{125}{1000} \times V \times \text{d.f.}$$

where O.D._{obs.} = optical density of test solution,

O.D._{st.} = optical density of 125 $\mu\text{g/ml}$ bovine serum albumin,

d.f. = final volume (ml) of sodium hydroxide in which sample was dissolved $\div$ volume (ml) of homogenate taken for assay,

V = volume of total homogenate (ml).

Thus, for 3 ml homogenate, 0.5 ml of which was diluted to 4 ml with sodium hydroxide, if optical density = 0.280, then total protein = $\frac{0.280}{0.237} \times \frac{125}{1000} \times 3 \times \frac{4}{0.5}$
= 3.6 mg.

Sample calculation for assay of alkaline phosphatase specific activity:

$$\text{Specific activity } \frac{(\mu\text{g/hr})}{(\text{mg})} = \frac{\text{O.D.}_{\text{obs.}}}{\text{O.D.}_{\text{st.}}} \times 40 \times V \times$$

$$\text{d.f.} \times \frac{60}{t} \times \frac{1}{p}$$

where $\text{O.D.}_{\text{obs.}}$ = optical density of test solution,

$\text{O.D.}_{\text{st.}}$ = optical density of 40 $\mu\text{g/ml}$ phenolphthalein

V = volume of total homogenate (ml),

d.f. = final volume (ml) of homogenate from which 0.5 ml test sample was taken $\div$ volume (ml) of original homogenate in dilution sample,

t = time (minutes of incubation)

p = amount of protein (mg) in total homogenate.

Thus, for 3 ml homogenate, 0.25 ml of which was diluted to 5 ml with distilled water, if optical density = 0.207 after 31.75 minutes of incubation, then, specific activity = $\frac{0.207}{0.360} \times 40 \times 3 \times \frac{5}{0.25} \times \frac{60}{31.75} \times \frac{1}{3.6} = 7.36 \frac{\mu\text{g/hr.}}{\text{mg}}$.