# Functional Differentiation of the Chick Endocrine Pancreas

## II. THE ALPHA CELLS AND GLUCAGON [1,2]

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*ABSTRACT* A radioimmunoassay for glucagon, together with electron microscopic observations of early embryonic alpha cells were utilized to examine developmental aspects of glucagon accumulation and release in the chick embryo. Immunoreactive glucagon was detected in both the pancreas and blood plasma from the fifth embryonic day onwards. In addition, emiocytotic events were observed in alpha cells as early as the fifth embryonic day. The early appearance of glucagon and its subsequent developmental profile correlate well with major events in carbohydrate metabolism occurring in the embryonic chick, and are discussed in relation to a functionally responding system, the developing liver. The present data show that glucagon is secreted at earlier embryonic stages than hitherto demonstrated, and suggest a developmental role for glucagon in hepatic glycogen metabolism.

The pancreas in the chick begins forming on or about the third embryonic day from three distinct endodermal primordia which evaginate from the gut in close proximity to the liver anlage (Romanoff, '60; Przbylski, '67). The single dorsal primordium subsequently fuses with the two ventral diverticula to form the definitive pancreas, which eventually differentiates into the four-lobed structure characteristic of the adult bird (Mikami and Ono, '62). The endocrine elements of the avian pancreas are contained in alpha ("dark") islets, composed of alpha and delta cells, and beta ("light") islets which consist of beta and delta cells. It is known that alpha and beta cells elaborate glucagon and insulin, respectively, while the hormonal role of the delta cell has yet to be fully defined.

Until recently, it had been assumed that the chick embryonic alpha cell began to differentiate well after the appearance of the pancreatic rudiment. The initial appearance of tinctorially distinct alpha granules in light microscope preparations has been reported on days 8 (Villamil, '42; Ghiani, '56), 12 (Potvin and Aaron, '27; Sandstrom, '34) and 15 (Lievre, '57; Grillo, '61). Grillo ('61) also measured "glucagon-like" activity based on the activation of hepatic glycogen phosphorylase in tissue extracts of chick embryonic pancreas and could detect its presence only from the thirteenth embryonic day onwards. With the electron microscope, however, Dieterlen-Lievre ('63) and Przybylski ('67) observed that alpha secretory granules were present in the 3-day pancreatic anlage, and Przybylski ('67) noted that granule release may occur as early as the fifth embryonic day. In addition, the immunocytological studies of Beaupain and Dieterlen-Lievre ('74) demonstrated that these early alpha granules contained glucagon.

The development of radioimmunological techniques for the detection of polypeptide hormones has made possible the measurement of very low concentrations of these substances in blood plasma and in tissue extracts. The application of such techniques to problems in embryonic development has already begun to shed new light on the functional significance of embryonic hormones in the control of growth and differentiation in developing cells (Rall et al., '73; Benzo and Green, '74). In the present study we have taken advantage of recent refinements in the radioimmunoassay for glucagon as part of a coordinated biochem-

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[2] A preliminary report on a portion of this work has appeared elsewhere (Benzo and Stearns, '75).

ical-morphological approach to the investigation of developmental aspects of glucagon storage and release in the chick embryo.

## MATERIALS AND METHODS

### *Animals*

Eggs from White Leghorn chickens (*Gallus domesticus*) were incubated at 38°C in a relative humidity of 50%. On appropriate days, embryos were removed to individual 15 × 100 mm Petri dishes, and were staged for study according to the Hamburger-Hamilton ('51) series. Observations reported for chicks at hatching were determined from material collected within one hour after egg emergence. Adult birds were 20-months-old when killed and had been maintained on commercial chick mash.

### *Tissue collection and preparation*

Animals were bled by cardiac puncture, and blood was drawn into ice-chilled, heparinized capillary pipets containing 500 KIU (Kallikrein Inactivator Units) of the proteinase inhibitor, Trasylol (FBA Pharmaceuticals), 50 $\mu$l of 1.0 M benzamidine hydrochloride, and 1.2 mg $Na_2$ EDTA per ml of collected blood. Blood from two to six animals from each stage was pooled, and plasma was obtained immediately by centrifugation and was stored at − 20°C for subsequent glucagon determinations.

Whole embryonic pancreases and randomly selected portions of the adult organ were frozen separately in glass tubes immersed in acetone/dry ice. Tissue extracts were prepared by sonication for ten seconds at 2°C in glass-distilled water containing 1000 KIU Trasylol. Protein in the pancreatic extracts was determined by the method of Lowry et al. ('51), with bovine serum albumin as a standard. Pancreatic tissue from two to four animals from each stage was pooled for each glucagon determination.

### *Immunoreactive glucagon determination*

Glucagon was determined in both plasma and pancreatic extracts according to the radioimmunological method developed in the laboratory of Dr. Roger Unger (The University of Texas Southwestern Medical School, Dallas, Texas). Antiserum (30K) highly specific for pancreatic glucagon was obtained from the same source. Glucagon-$I^{125}$ (specific activity, 514 mCi/mg) was purchased from Nuclear Medical Laboratories (Dallas, Texas), and crystalline beef-pork glucagon standard was kindly provided by Dr. William Bromer of Eli Lilly and Company. For each glucagon determination, three dilutions were made of each plasma or pancreatic sample, and each dilution was assayed in duplicate.

The behavior of glucagon in the present immunoassay is defined by the appropriate concentrations of beef-pork glucagon standard. The glucagon values reported herein represent the amount of chicken glucagon which is immunologically equivalent to beef-pork glucagon. Recently, Kimmel (cited by Hazelwood, '73) has found that both the amino acid composition and the sequence of residues of chicken glucagon are the same as those which have been reported for beef, pork and rat glucagon, except for a replacement of asparagine by serine at position 28. Thus, virtually identical molecules compete for the glucagon antibody in the assay system. Furthermore, it has been demonstrated that the dilution curves (plots showing the effects of increasing dilutions of unlabelled beef-pork glucagon and of tissue extracts on the percentage binding of labelled beef-pork glucagon by rabbit anti-beef-pork glucagon antiserum) of pancreatic extracts or plasma from chickens or ducks are parallel to those of crystalline beef-pork glucagon, indicating that these avian preparations are immunologically similar to beef-pork glucagon with respect to their cross-reactivity with antibodies (Assan et al., '69; Leclercq-Meyer et al., '70).

Values are presented as mean ± standard error of the mean (SEM). Mean values between consecutive age groups were compared by Student's t-test. Differences with P-values $< 0.05$ were considered significant.

### *Electron microscopy*

Pancreatic tissue was fixed for two hours at 4°C in 1% osmium tetroxide buffered at pH 7.4 with veronal acetate, dehydrated by increasing concentrations of ethanol, and embedded in Araldite 502 by standard procedures (Pease, '64). The resin blocks were sectioned on a Porter-Blum MT2-B ultramicrotome using glass or duPont dia-

TABLE 1

*Pancreatic and plasma glucagon concentrations in the developing chick*

| Age (embryonic days) | Pancreatic glucagon (ng/mg pancreatic protein) | P-value | Plasma glucagon (pg/ml) | P-value |
|---|---|---|---|---|
| 5 | 10.45 ± 0.67 (11) | | 149.17 ± 12.39 (6) | |
| | | < 0.001 | | N.S. |
| 8 | 62.28 ± 7.21 (9) | | 160.00 ± 11.55 (4) | |
| | | N.S. | | < 0.001 |
| 11 | 92.00 ± 16.22 (5) | | 257.50 ± 13.27 (6) | |
| | | < 0.05 | | < 0.001 |
| 14 | 37.30 ± 10.73 (5) | | 120.79 ± 21.34 (7) | |
| | | N.S. | | < 0.05 |
| 16 | 40.28 ± 3.77 (6) | | 210.83 ± 31.89 (6) | |
| | | N.S. | | N.S. |
| 18 | 42.90 ± 2.60 (5) | | 230.30 ± 27.25 (5) | |
| | | N.S. | | N.S. |
| 20 | 49.45 ± 5.13 (4) | | 271.25 ± 42.14 (4) | |
| | | < 0.001 | | < 0.001 |
| Hatching | 4.91 ± 1.12 (4) | | 822.57 ± 75.77 (7) | |
| | | N.S. | | N.S. |
| Adult | 6.71 ± 1.76 (4) | | 863.0 ± 171.2 (5) | |

Values are means ± SEM with the number of determinations in parentheses. Pancreatic tissue or blood from two to six animals was pooled for each determination. P-values refer to differences in pancreatic or plasma glucagon between consecutive age groups. N.S. = not significantly different at $p < 0.05$.

mond knives. Ribbons of sections showing pale-gold to silver-gray interference colors were collected on uncoated 300-mesh copper grids and stained with uranyl acetate followed by lead citrate. Sections were examined with an RCA EMU-3F electron microscope.

## RESULTS

### *Glucagon content of the developing pancreas*

Immunoreactive glucagon was present in the pancreas of the 5-day chick embryo (table 1). Pancreatic glucagon concentration increased markedly ($p < 0.001$) between the fifth and eighth embryonic days, did not change significantly between days 8 and 11, and decreased ($p < 0.05$) by the fourteenth day. The glucagon content remained constant through day 20, then dropped precipitously ($p < 0.001$) at hatching. This developmental pattern of glucagon accumulation contrasts with the biphasic profile previously observed for the accumulation of insulin in the chick pancreas (Benzo and Green, '74). Pancreatic glucagon concentration in the neonatal chick approximated that found in the adult bird. Although, as noted below, the level of circulating glucagon rose nearly three-fold at hatching, the release of pancreatic glucagon during this period could account for only a small fraction of the difference in the pancreatic glucagon concentration observed between that in the 20-day embryo and that in the hatched chick. Such a decrease in glucagon specific activity in the neonatal pancreas more appropriately reflects a dramatic increase in the protein concentration of the exocrine cells, by far the predominant cell type in this tissue (Romanoff, '60).

### *Plasma glucagon concentration*

Plasma glucagon levels were constant through the eighth embryonic day, peaked sharply ($p < 0.001$) by day 11, then dropped ($p < 0.001$) by the fourteenth day (table 1). An increase ($p < 0.05$) in the concentration of circulating glucagon occurred between the fourteenth and sixteenth days. Plasma glucagon levels then remained relatively steady through the twentieth day and increased dramatically ($p < 0.001$) at hatching. The plasma glucagon concentration in the newly-hatched chick was comparable to that of the adult chicken and is within the range of that value reported for the adult duck (Leclercq-Meyer et al., '70).

### *Morphological observations*

In the present study, morphological observations of the developing alpha islet were utilized primarily to provide complementary evidence for glucagon content as well as other indications of precocious alpha cell secretory activity. At the ultra-

structural level, the chick alpha cell can be distinguished by its round to oval nucleus, which usually contains one or more nucleoli, a relative paucity of mitochondria and rough endoplasmic reticulum and a prominent Golgi complex which often contains moderately electron-opaque prosecretory material. The mature alpha secretory granules are round or oval, and are approximately 250 to 300 nm in diameter. Characteristically, each granule displays an electron-opaque core surrounded by a limiting membrane with an intervening electron-lucent space or halo. Since more detailed morphological analyses of the developing chick alpha cell have appeared (Pryzbylski, '67; Beaupain and Dieterlen-Lievre, '74), our observations were limited to the early (5-day) embryonic period.

Figures 1–3 show portions of alpha cells from 5-day chick embryonic pancreas. These cells contained many well-developed secretory granules which were found scattered throughout the cytoplasm, as well as immediately subjacent to the plasma membrane. The early alpha cells were already associated with a juxta insular capillary network and secretory functional activity was evidenced by the presence of emiocytotic images in the alpha cell at the capillary-islet interface. In addition, figure 3 shows a number of alpha granule cores which have a "fuzzy" periphery. Although some feel that such images may be artifactual (Pictet and Rutter, '72), others contend that these may represent part of an alternative, or at least a supplementary, secretory process, namely intracytoplasmic dissolution of the hormone-containing granule (Munger, '62; Rhoten, '71, '73; Smith, '75).

## DISCUSSION

The most convincing evidence of functional activity of an endocrine gland is not only the presence of its secretory product(s) within the gland but the identification of its hormone(s) in the blood, as well. Our present findings not only verify previous speculations regarding the early functional potential of the chick endocrine pancreas but, and more importantly, show also that glucagon is indeed secreted from the early embryonic alpha cells and that the hormone is precociously available to target tissues during organogenesis. In this regard, correlation of these aspects of functional maturation is desirable to better understand the developmental interrelationships between the chick endocrine pancreas and responding systems, particularly in view of the belief that, unlike in mammals, glucagon may be the dominant pancreatic hormone controlling avian intermediary metabolism (Hazelwood, '73).

Recently radioimmunological and ultrastructural evidence was reported for the production and secretion of insulin by 5-day chick embryo beta cells (Benzo and Green, '74). The early appearance and subsequent developmental profile of insulin correlated well with changes *in ovo* associated with hepatic glycogen metabolism, and suggested a possible role for insulin in hepatic glycogen synthesis. Similarly, the present results suggest an early developmental role for glucagon in embryonic glycogen metabolism. Our observed increase in the plasma glucagon level between the eighth and eleventh embryonic days is accompanied by an increase in hepatic glycogen phosphorylase *a* activity (Benzo and de la Haba, '72) prior to the marked decrease in liver glycogen on days 11–12 (Croisille and Le Douarin, '65). In addition, our present findings of lower levels of pancreatic and plasma glucagon by day 14 correspond in time with increases in plasma insulin (Benzo and Green, '74), hepatic glycogen deposition (Croisille and Le Douarin, '65), decreases in liver phosphorylase *a* activity (Benzo and de la Haba, '72) and decreases in plasma glucose values (Benzo and Green, '74).

Just prior to hatching, plasma glucagon is seen to rise markedly, concurrent with sharp decreases in liver glycogen (Croisille and Le Douarin, '65), hepatic glycogen synthetase activity (Grillo et al., '64; Benzo and de la Haba, '72) and plasma insulin (Benzo and Green, '74). During this same period, liver phosphorylase is at its highest embryonic activity (Grillo et al., '64; Benzo and de la Haba, '72) and the plasma glucose concentration rises sharply (Benzo and Green, '74). In addition, our recent studies on developmental alterations in the level of cyclic AMP in chick liver have yielded data which show that a significant,

positive correlation ($r = 0.986$; $p < 0.01$) exists between hepatic cyclic AMP accumulation and circulating glucagon levels in the embryonic chick (Benzo and Stearns, unpublished). These preliminary findings are in good agreement with the developmental fluctuations in plasma glucose and insulin levels, liver glycogen concentration and hepatic activities of glycogen synthetase and phosphorylase noted above, and are consistent with the metabolic alterations which occur following the response in vivo of hepatic cyclic AMP to glucagon in the adult bird (Frohlich and Marquardt, '72). These observations strongly suggest that glucagon may also play such a regulatory role in embryonic glycogen metabolism.

Although embryonic days 10 to 14 have often been cited as the beginning of functional integration between the endocrines and their responding tissues in the chick (Croisille and Le Douarin, '65), it is clear from the present and other recent work that the endocrine pancreas produces and releases both glucagon and insulin much earlier during development than previous histological or bioassay techniques had indicated, and that tissue carbohydrate levels may be regulated in part by such hormonal influence over metabolic processes very early in embryogenesis.

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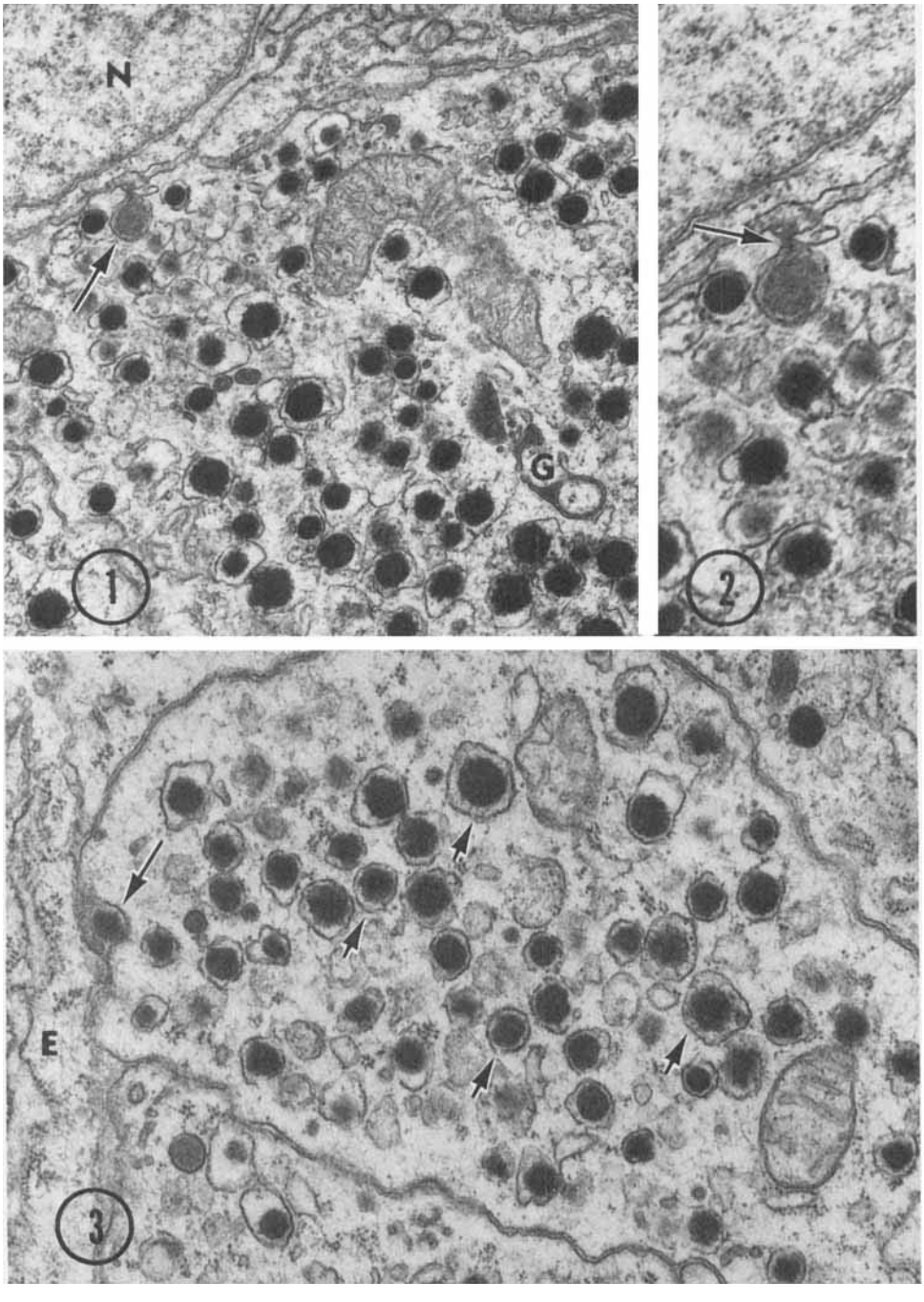
## PLATE 1

### EXPLANATION OF FIGURES

1 Electron micrograph from 5-day chick embryo pancreas. The micrograph shows a portion of an alpha cell with many well developed secretory granules containing electron-opaque cores, and with an apparently active Golgi complex (G). An alpha granule in emiocytosis (arrow) can be seen at the capillary-islet interface, along with a portion of the nucleus (N) of an endothelial cell. × 17,000.

2 An enlargement of the upper left portion of figure 1 showing what was interpreted as an emiocytotic event. The arrow points to the coalescence of an alpha granule's limiting membrane with the plasma membrane. The granule core is less electron-opaque than those of its neighbors, while the usually electron-lucent space between the granule core and its limiting membrane appears to have an increased opacity. × 30,800.

3 Electron micrograph of portions of 5-day chick embryo alpha cells. The micrograph shows a number of secretory granules (short arrows) with cores that appear to have a fuzzy periphery and which show regions of moderate electron-opacity between the granule cores and their limiting membranes. Such images suggest that in addition to granule extrusion by emiocytosis (long arrow), hormonal material might be released by intracytoplasmic dissolution of the granules, as well. A portion of an endothelial cell (E) can be seen at right. × 24,000.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