STUDIORUM PROGRESSUS

Immunohistochemical and Ultrastructural Studies on the Endocrine Polypeptide (APUD) Cells of the Avian Gastrointestinal Tract

In recent years a considerable number of studies have been devoted to the cytological, cytochemical and ultrastructural characteristics of endocrine cells in the mammalian gastrointestinal tract. These cells are responsible for the production of a variety of polypeptide hormones[1–11], and one amine. With the exception of the enterochromaffin (EC) cells [12–14], the endocrine cells of the avian gut have received a good deal less attention. In the case of mammalian gut they have been shown to belong to the APUD series of polypeptide hormone-producing cells[15,16] from whose cytochemical 'amine-handling' characteristics the name APUD (Amine Precursor Uptake and Decarboxylation) is derived.

It has been postulated[17–19] that all the endocrine cells of the APUD series are descendants of neuroectodermal stem cells arising in the neural crest. This view is opposed, in the case of the endocrine cells of gut and pancreas, by the classical theory that all are of entodermal (foregut) origin.

Identification of individual gut endocrine cells with the production of individual hormones has been carried out, for the most part, by correlation of the distribution of specific immunofluorescence with the distribution of cell types recognizable by their ultrastructure. Since we possessed antisera to a number of peptides of gastrointestinal origin, we were disposed to follow the lead given by KETTERER et al.[20] on the distribution of gastrin in the gut of the chicken by attempting to localize the cellular sources of gastrin, enteroglucagon, secretin, vasoactive intestinal peptide (VIP) and gastric inhibitory peptide (GIP) in the avian gastrointestinal tract.

Material and methods. We used 2 species of birds: 35 quails (*Coturnix coturnix japonica*), and 19 White Leghorn chickens, whose ages ranged from newly hatched to 3 months old. The birds were killed by administration of ether and samples were taken immediately from proventriculus, gizzard, duodenum (first part), duodenum (second part), small intestine (first part), small intestine (second part), large intestine, caecum and colon. Small pieces, from each of the above regions, were processed by each of the procedures outlined below.

Immunohistochemistry. Fixation for immunological studies was carried out in 3 different ways: 1. Some samples were fixed in methanol-free formaldehyde[21] for 3–4 h, then washed overnight in 0.1 *M* phosphate buffer saline containing 7.5% sucrose. Afterwards each sample was divided into 2 portions, one being quenched in Arcton (Freon) 22 at −158 °C, for subsequent cryostat sectioning, and the other dehydrated in a graded series of alcohols, cleared in xylene and embedded in paraffin wax.

2. Other samples were fixed in a 4–10% solution of a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, in 0.1 *M* phosphate buffer[22]. After washing in buffer sections were cut on a cryostat.

3. Yet other samples were quenched in Arcton (Freon) 22 at −158°C and freeze-dried for 12–24 h at −40 °C in a thermoelectric freeze dryer. After removal, the dried blocks were incubated for 3 h at 55 °C in a sealed vessel containing diethylpyrocarbonate (DEPC). They were subsequently embedded in paraffin wax blended with

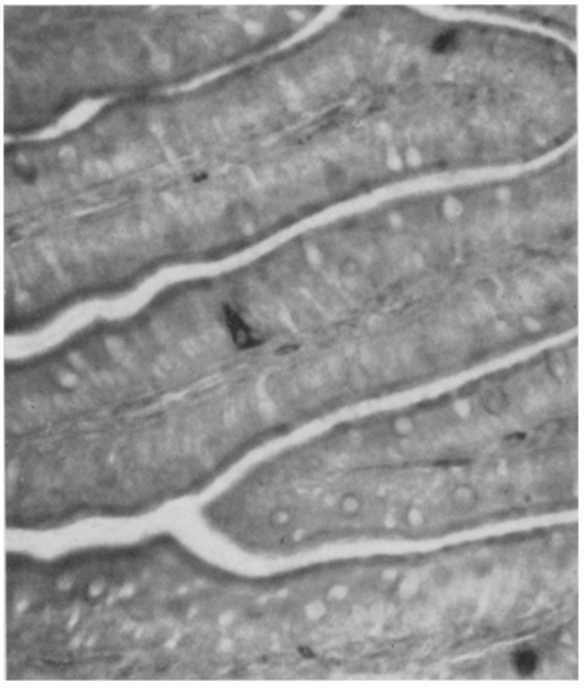

Fig. 1. Quail jejunum. Peroxidase technique shows a cell reacting with anti-gastrin serum containing hormone on both sides of the nucleus. ×350.

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synthetic plastic co-polymers (m.p. 58 °C). The use of DEPC as a vapour phase fixative for freeze-dried tissues has been described by PEARSE et al[23].

In all cases cryostat sections were picked up directly from the knife on to gelatine-formaldehyde coated slides. Paraffin sections were picked up from water on to albuminized slides and dried, overnight, at 37 °C.

An indirect method for immunofluorescence[24] was used, employing for the first layer antisera to each of the following hormones: gastrin (synthetic human), caerulein (synthetic), glucagon (porcine), secretin (porcine), VIP (porcine), GIP (porcine), and for the second layer, fluorescein-labelled goat anti-rabbit and anti-guinea-pig IgG sera (Hyland). As controls we used 1. normal rabbit serum, 2. second layer alone and 3., where possible, the first layer serum absorbed with the pure antigen.

Peroxidase-labelled antisera (donkey anti-rabbit IgG and rabbit anti-guinea-pig IgG) were prepared using peroxidase type VI (Sigma) bound to the globulin by the glutaraldehyde method[25]. Peroxidase activity was demonstrated by the method of GRAHAM and KARNOVSKY[26].

Preparation of antisera. The antisera used were raised in LOP rabbits by repeated injection of antigens coupled to bovine serum albumin by the carbodiimide condensation reaction. The product was purified by gel filtration, in order to remove the low molecular weight unreacted antigen. The latter is likely to induce states of partial tolerance in the recipient animal with resulting production of low titre, low avidity antibody.

After several months of immunization, with total doses of pure antigen ranging from 200 µg to 2 mg., high titre antisera were produced in many of the animals.

Specificity of antisera. We tested this by 2 methods. First, by measuring the displacement of labelled antigen from its antibody by a different antigen. For example, glucagon was tested against gastrin, or secretin against caerulein. Secondly, we measured the ability of antigen to bind radioactive antibody to a non-related antigen.

Fluorescence microscopy was carried out using a Zeiss (Oberkochen) Standard Universal microscope with HBO 200 lamp BG12 and BG38 excitation filters and a K530 barrier filter (50% transmission at about 530 nm) and with a Leitz Orthoplan microscope fitted with a HBO 100 lamp and XBO 75 lamps and a Ploem illuminator. FITC was excited at 490 nm, using 2 × KP490 interference filters and TK510 dichroic mirror, and a K515 barrier filter was employed. Photomicrographs were taken on Ilford FP4 film.

Amine-precursor uptake, decarboxylation and storage. The formaldehyde induced fluorescence method[27] was used to detect the presence of intrinsic amines, and also extrinsic amines after administration of L-3,4-dihydroxyphenylalanine. 3 chicks and 3 adult quails were injected i.p. with 100 mg/kg of L-DOPA and killed 1 h later. An equal number of birds acted as controls.

Small pieces of gastrointestinal mucosa were subjected to the freeze-drying, formaldehyde vapour routine (−40 °C for 16 h; 60 °C or 80 °C for 4 h). FIF was assessed using exciting light at 405 nm (AL 405 interference with supplementary glass filters and TK 455 dichroic mirror) and a K470 barrier filter.

Optical microscopy. Small blocks from each region were fixed for 24 h in 6% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.4) and in Bouin's fluid. All blocks were subsequently washed in water, dehydrated, cleared and embedded in 56 °C paraffin wax. Sections (5 µm) from appropriately fixed blocks were processed by methods designed to demonstrate the specific secretory granules of the various endocrine cells. These were: lead heamatoxylin[28], the DMAB method for tryptophan[29], the Masson-Fontana method for enterochromaffin granules[30], and a silver impregnation for argyrophilia[31].

Electron microscopy. Small blocks of tissue were fixed, immediately after removal, in 3% glutaraldehyde in 0.1 *M* phosphate buffer at pH 7.2 for 2 h at 4 °C. Excess fixative was removed from the samples by repeated washing in phosphate buffer containing 0.1 *M* sucrose. They were then dehydrated in ethanol and epoxypropane and finally embedded in Araldite mixture. With some blocks a double fixation procedure was carried out, using post-fixation with osmium tetroxide at 4 °C for 2 h. Sections were stained by lead citrate and uranyl acetate and viewed in AEI 6B and Corinth 275 microscopes.

Results. Histology. It appeared that the majority of endocrine cells, throughout the length of the gastrointestinal tract, were stained by the lead haematoxylin technique. They were also argyrophil with the Grimelius method. In all regions the cells were sitated predominantly in the basal area of the glands. Argentaffin cells (Masson-Fontana) were scarce in the first portions of the gut (proventriculus, gizzard) but numerous in the mid and lower portions. These cells were positive with the DMAB method.

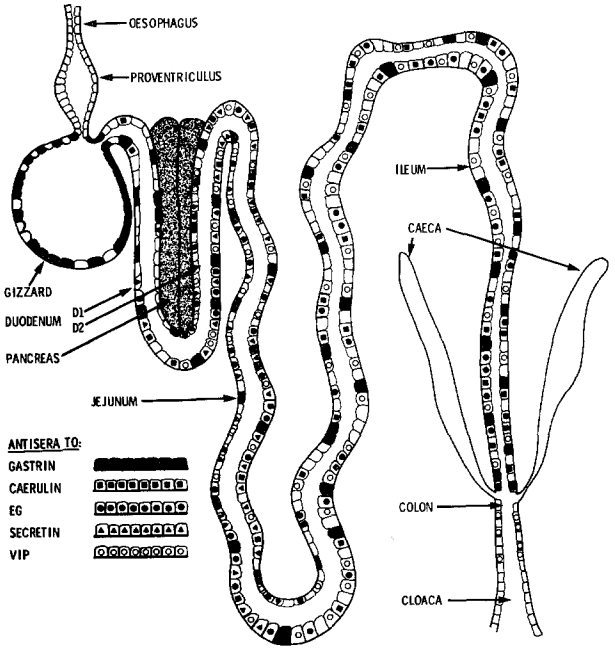

Fig. 2. Immunohistochemical localization of polypeptide hormones in the avian gastrointestinal tract.

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APUD-FIF. Cells displaying an intense fluorescence were observed in all regions of the gut. Those with a yellow fluorescence (E_{max} 520 nm) were easily distinguished from the usually greater number with the green fluorescence of dopamine (E_{max} 470 nm). The distinction and the identity of the fluorophores, was checked by microspectrofluorometry. In the control tissues only yellow fluorescent cells could be seen.

Immunohistochemistry. Cells reactive with antisera to the following hormones were found: gastrin, caerulein, secretin, VIP, glucagon. No cells reacting with antisera to GIP were observed. When localized in the basal part of the glands the reacting cells, whatever hormone they contained, were predominantly triangular in shape with a long apical process reaching, occasionally, to the gland lumen. When present in the villi they were inclined to be fusiform, or oval. Cells reacting with antisera to gastrin and caerulein were present in all regions except the proventriculus and the caeca (Figure 1). No other sera gave positive results in gizzard or caeca but secretin immunoreactive cells were restricted to the duodenum and first portion of the jejunum. Enteroglucagon cells were predominantly localized in the second part of the duodenum and in jejunum and ileum. They were not found in colon or caeca, nor in the gizzard or first part of the duodenum. VIP cells were present in all regions except the gizzard.

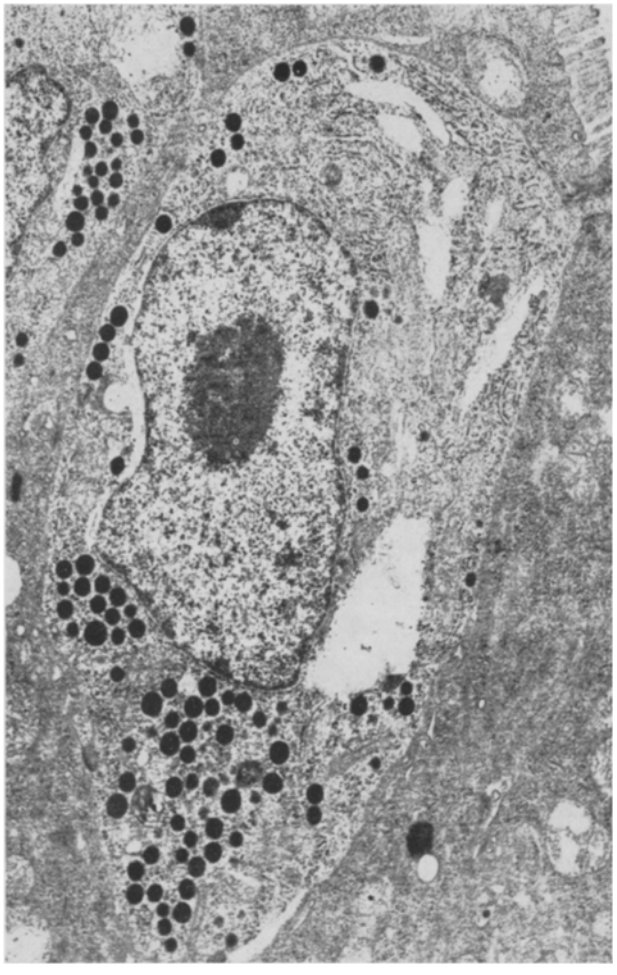

Fig. 3. Quail jejunum. Glutaraldehyde/osmium fixation, lead citrate/uranyl acetate counterstaining. This non-enterochromaffin endocrine cell has round granules (average diameter 150–250 nm) situated on both sides of the nucleus. This cell reaches the gland lumen into which its terminal tuft of microvilli projects. ×10500.

Preliminary studies to determine the precise cell of origin for the various hormones, using sequential staining or serial section techniques, indicated that, as in mammals, glucagon and secretin are stored in different cells. Clear distinction could not be made between glucagon and gastrinreacting cells, however. Gastrin-reacting and caerulein-reacting cells appeared to be identical.

When the immunohistochemical preparations were post-fixed in 6% glutaraldehyde all the endocrine cells were reactive with lead haematoxylin and Grimelius silver (argyrophilia). They were not stained by the Masson-Fontana technique (argentaffinity) indicating that none of the hormones identified in the gut were the product of enterochromaffin cells.

The results achieved with the peroxidase-labelling technique were essentially the same as with immunofluorescence. The combined results of both methods are illustrated in the text figure (Figure 2) below.

Ultrastructural Studies. Endocrine cells with characteristic electron-dense secretory granules (Figure 3) were found in all regions of the gut. Some, possessing polymorphic, highly electron-dense granules, were identified as EC cells. Others, in the duodenum, contained small round granules with a thin halo between content and membrane. These resembled the S cells of the mammalian (Wiesbaden) classification.

Discussion. Gastrin was extracted from chicken upper intestine, and tested by bioassay in the cat[32]. Using radioimmunoassay, its distribution was investigated by KETTERER et al[20]. who found it in the duodenum alone. Glucagon was extracted from the intestine of the duck and chicken by SAMOLS et al.[33], ASSAN et al.[34], KRUG and MAIHLE[35] and KRUG et al.[36]. It was identified by radioimmunoassay and its mol. wt., in the duck, as estimated on Sephadex G25, was 7000 daltons[35].

Secretin was extracted from the upper intestine of the chicken[32] and VIP, more recently, was isolated from chicken intestine[37]. There are no reports of the study of GIP in birds.

Our findings, (illustrated in Figure 2), are in general agreement with the above data. If possible they should be integrated with the few recorded observations on the histology and cytochemistry of avian gut endocrine cells, reviewed by GABE[14,38] and with their recorded ultrastructure[13]. We are unable, however, to fit these cells into the Wiesbaden classification and thus to equate cell types with specific hormones. The most we can say is that none of the hormones demonstrated is present in the EC cells, and that all are present in argyrophil cells. No further breakdown is possible.

In mammalian tissues, after formaldehyde fixation, only pancreatic and not gut glucagon cells react with antisera to pancreatic glucagon. In the bird we found that the EG cells of the gut were still strongly reactive after

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this fixative. We conclude that in avian species there is a closer relationship between the two glucagons than is the case with mammals.

Finally, our data on the distribution of the 4 'hormones' gastrin, glucagon, secretin and VIP, in the avian gastrointestinal tract can form a basis for investigations into their physiology. This field is presently somewhat neglected.

[39] For their valuable help, in supplying peptide hormone antigens and antisera, or in raising and testing such antisera, we would like to thank Prof. V. MUTT, Drs S. R. BLOOM and S. I. SAID, and also Farmitalia. Miss C. GREEN provided excellent technical assistance. Grants from the Medical Research Council (microspectrofluorometer), the Wellcome Trust, the Ernest and Minnie Dawson Trust and the Volkswagenwerk Stiftung made the work possible.

Resumen. Estudios con immunofluorescencia realizados en al tracto gastro-intestinal de las aves (pollo y codornix) han demostrado el origen celular y la distribución de cuatro hormonas polipeptídicas: Gastrina, Secretina, Enteroglucagon y VIP. Las cuatro hormonas están presentes en células argirófilas y no en las células argentafines (células enterocromafines).

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4 February 1974.

COGITATIONES

'...We said that the offspring should come from parents in their prime.' 'True'. 'Do you agree that the period of the prime may be fairly estimated at 20 years for a woman and 30 for a man?' 'How do you reckon it?' he said. 'The women,' I said, 'beginning at the age of 20, shall bear for the state to the age of 40, and the man shall beget for the state from the time he passes his prime in swiftness in running to the age of 55.'

PLATO, (*The Republic*, Harvard University Press 1953), book V, p. 465.

On Correlation between the Generation Age of the Fathers and Grandfathers and the Intelligence of the Descendants[1]

1. *The problem.* Which prerequisites could play a rôle in the development of intelligence? Is it genetically inherited, the effect of the social environment, or something else that has until now not been considered? The following study started from the assumption that the well-known postulate of PLATO[2] and ARISTOTLE[3] that men should not reproduce before the age of 30, was probably based on extended observations. In the same context belong some data on families ascending or descending in intellectual endowment. Since a definite change of status is very often first observed after 2 generations, the generation age[4] of the grandparents should also be taken into consideration. We define therefore a characteristic '*Paternal Trinomial*' (Dreierzahl) by the formula

$$S_f = F + Z_f + Z_m$$

where F is the age of the father and Z_f, Z_m the ages of the paternal and maternal grandfathers, all at the time of corresponding generation.

Analogously the '*Maternal Trinomial*' is defined by

$$S_m = M + Y_f + Y_m$$

where M is the age of the mother and Y_f, Y_m the ages of the grandmothers, all at the time of corresponding generation.

2. *Series of investigations.* In order to avoid the influence of an intentional selection, I used first only lists of persons which were brought together by other authors, and studied the generation age of the fathers and grandfathers in these series completely and without any exception. Three of these series consist of highly gifted personalities, one series of under average endowed persons. In this way we discussed the two extreme cases of endowment. In order to consider the problem in the most general way, we then studied the interconnection between the level of endowment characterized by the intelligence quotient (IQ) and the ages of parents and grandparents, using a sample obtained specially for that purpose.

2. 1. In the series of monographs edited by ROWOHLT[5], we have a number of biographies of outstanding personalities where, however, the choice was made according to the anticipated interest of the reader. In 109 subjects we could find the age of the father in 96 cases[6], and in 39 cases the paternal trinomial could also be obtained. In the result, 15.6% of the fathers were under 30, while no paternal trinomial was under 90[7].

[1] Dedicated to ALEXANDER M. OSTROWSKI on his 80th birthday.

[2] 'The limit of the marriage-age shall be from 16 to 20 years – the longest time allowed – for a girl, and for a boy from 30 to 35.' PLATO, *Laws*, (Harvard University Press, Cambridge, Mass. 1952), book VI, p. 501.

[3] 'The body (of men) is most fully developed from 30 to 35 years of age, the mind at about 49.' ARISTOTLE, *The 'Art' of Rhetoric* (Harvard University Press, Cambridge, Mass. 1959), vol. II, p. 257.

[4] Since the fecondation age is not easy to find with some precision, we use instead the generation age that is the age of the parents at the birth of the child.

[5] *Rowohlt Monographien*, volumes 1–116 edited 1959–1971. We did not use vol. 33, 34, 35, 55, 56, 69 and 70 since they do not contain indications about single personalities.

[6] In this series we find also personalities like THOMAS OF AQUINAS, FRANCIS OF ASSISI, ARISTOTLE and JOHN THE BAPTIST (although in the case of the latter we have in the Bible the indication of a high age of his father) but in these cases we cannot expect to find the life data of their fathers.

[7] I was privileged to submit these results in summer 1971 to Professor ADOLF BUTENANDT and Professor GUSTAV WAGNER; I am indepted to both for valuable indications concerning my future work.