Department of Pathology,¹ University of Umeå, Umeå, Sweden; Department of Anatomy,²
University of Minnesota School of Medicine, Minneapolis, U. S. A.; Departments of
Histology³ and Biochemistry,⁵ University of Uppsala, Uppsala, Department of
Endocrinology,⁴ Karolinska Hospital, Stockholm,
and
the Kristineberg Marine Biological Station,⁶ Fiskebäckskil, Sweden

**Some Phylogenetical Aspects on the Occurrence of Somatostatin
in the Gastro-Entero-Pancreatic Endocrine System.
A Histological and Immunocytochemical Study, Combined
with Quantitative Radioimmunological Assays of
Tissue Extracts***

Sture FALKMER,¹ Robert P. ELDE,² Claes HELLERSTRÖM,³
Birger PETERSSON,³ Suad EFENDIĆ,⁴ Jan FOHLMAN,⁵
and
Jan-Bertil SILJEVALL⁶

Summary. Rodioimmunoassayable somatostatin (SRIF) was found in acid ethanol extracts from various parts of the gastro-entero-pancreatic (GEP) endocrine system in reptiles, amphibians, teleost bony fish, cartilaginous fish, and jawless fish, as well as in a deuterostomian invertebrate, the tunicate, *Ciona intestinalis*. The cellular sites could, as a rule, be easily visualized light-microscopically by the peroxidase-anti-peroxidase (PAP) immunocytochemical procedure, using guinea-pig and rabbit antisera against synthetic SRIF. The standard Hellerström-Hellman technique, used to detect argyrophil SRIF-storing D cells, failed to visualize the SRIF cells in the GEP endocrine system of the tunicate and of the jawless fish. Moreover, the results confirmed the previous description that this technique only exceptionally (and sometimes only after further modifications) gave positive results when applied to the GEP endocrine system of bony fish, amphibians, and reptiles. In cartilaginous fish, however, it worked adequately and confirmed the radioimmunological and immunocytochemical observations.

In the mucosa of the alimentary tract and in the parenchyma of its associated glands of one echinoderm and two pelecypod molluscs and one crustacean arthropod no signs of the occurrence of SRIF-storing cells were observed using the three correlated procedures. In several of these tissues, signs of the occurrence of insulin-producing cells had previously been observed. Thus, SRIF seems to appear at a later evolutionary stage than insulin.

The principal islets (Brockmann corpuscles) of the marine teleost fish, *Cottus scorpius*, had the highest concentrations of radioimmunoassayable SRIF of all the GEP organs and tissues investigated, viz. about 200 ng/mg wet weight. Nevertheless, it was only 1/5 of the actual insulin content.

The recent discovery that the hormone somatostatin (the somatotropin release inhibiting factor; SRIF) is present in a large number of locations in the central and peripheral nervous systems and in endocrine cells of the thyroid gland and the gastro-entero-pancreatic (GEP) endocrine system (see HÖKFELT et al., 1975; VALE et al., 1975; PEARSE et al., 1977) has been followed only to a limited extent by phylogenetical studies. As usual when a new hormone is being discovered, most of the original investigations were made in the conventional mammals and birds. It was

* This work was supported by grants from the Swedish Medical Research Council (Projects No. 12X-718 and 12X-109).

known early, however, that the SRIF in the nervous system occurs not only in endothermic vertebrates but also in amphibians and bony fish (DUBOIS et al., 1974). As to the presence of SRIF-storing cells in the GEP endocrine system of ectothermic animals, there are only a few recent reports, giving the actual tissue contents by radioimmunoassays and the immunocytochemical localization of SRIF-cells in bony fishes and cyclostomes (VALE et al., 1976; JOHNSON et al., 1976; VAN NOORDEN et al., 1977; see also FALKMER and ÖSTBERG, 1977). In addition, there are some biosynthetic studies on SRIF, using the islet parenchyma of a bony fish (NOE et al., 1977).

The aim of the present investigation is to make a more comprehensive and systematic phylogenetical study of the occurrence of SRIF, correlating the results of radioimmunoassays of the SRIF content of acid-ethanol extracts of various parts of the GEP endocrine system with those obtained after a conventional light-microscopical examination, using procedures known to stain the D (or A₁) cells* of homothermic vertebrates. To some extent, the light-microscopical observations were correlated also with the results of immunocytochemical investigations. In this way, an idea might be obtained as to whether SRIF is a phylogenetically young or old hormone, and whether conventional light-microscopical staining procedures are reliable when applied to a search for D cells in the GEP endocrine system in lower animals.

Material and Methods

Animals

The following animals were investigated.

Protostomian invertebrates:

Arthropoda:	The shore crab, <i>Carcinus maenas</i> (a crustacean decapode)
Mollusca:	The whelk, <i>Buccinum undatum</i> (a gastropode)
	The sea mussel, <i>Mytilus edulis</i> (a pelecypode)

Deuterostomian invertebrates:

Echinodermata:	The starfish, <i>Asterias rubens</i>
Chordata (Tunicata):	The sea-squirt, <i>Ciona intestinalis</i>
Cephalochordata:	The lancelet, <i>Branchiostoma lanceolatum</i> (the well-known "Amphioxus")

Vertebrates:

Agnatha:	
Cyclostomata:	The Atlantic hagfish, <i>Myxine glutinosa</i>
Gnathostomata:	
Chondrichthyes:	The spiny dogfish, <i>Squalus acanthias</i>
Osteichthyes:	The daddy sculpin, <i>Cottus scorpius</i> (a marine teleost)
Amphibia:	The frog, <i>Rana temporaria</i> (Anura)
Reptilia:	The grass snake, <i>Natrix natrix</i> (Squamata)
	The adder, <i>Vipera berus</i> (Squamata)

* In this report we have used the recently adopted Lausanne nomenclature for GEP endocrine cells (SOLCIA et al., 1977).

Sampling procedures

All animals used were adult, non-fasting, seemingly healthy individuals, of either sex (as far as the sex could be determined), usually sacrificed within a day or two after capture (unless specified otherwise). Except for the frogs and snakes, all the animals were obtained at the Kristineberg Marine Biological Station at Fiskebäckskil on the Swedish west coast. Most animals were caught during late summer and autumn.

The selection of organs and tissues to be dissected and analyzed was based on our previous work on the occurrence of insulin in these animals (WILSON and FALKMER, 1965; FALKMER and WILSON, 1967; DAVIDSON et al., 1971) and on data in the literature (FALKMER and ÖSTBERG, 1977). Small specimens from organs, specified below, were taken at random for histological and immunocytochemical investigations and fixed in freshly prepared Bouin's fluid overnight. Some specimens from snake pancreas were also fixed in Zenker's fluid. After rinsing in frequently changed volumes of 70% ethanol for several days, the specimens were dehydrated, cleared, and embedded in paraffin, cut, deparaffinized, rehydrated, and stained as further specified below. Most of the material collected at the dissections was, however, taken for radioimmunological SRIF assays. These specimens were rapidly minced with a pair of scissors, and freed from intestinal contents, blood, other body fluids and contaminants by briefly rolling the cut pieces on clean filter paper. The minced and blotted specimens were then rapidly transferred to a dry glass tube, standing in a Dewar flask, containing a mixture of dry CO₂ ice and 95% ethanol; the temperature was checked at regular intervals and kept between -40° and -60°C. When all the material had been collected, it was rapidly weighed and transferred to the deepfreeze (-30°C).^{*} The preparation of the extracts for SRIF assay is further described below.

From the **shore crabs**, samples were taken from the mucosa of the stomach, the mid-gut caecum, and the rest of the gut, as well as from the parenchyma of the hepatopancreas. Specimens from 21 crabs were pooled. The total amounts of the collected specimens varied from 1 to 2g, as further specified in Table 1. The crabs weighed between 35 and 50 g and were about 1/2 dm broad over the scutellum.

Specimens from the **whelks** and the **sea mussels** also were taken from the mucosa of the gut and the parenchyma of the hepatopancreas. In the whelks, specimens from the stomach and the protein gland were also included. Altogether, 17 whelks and 32 sea mussels were used. The whelks weighed about 25–40 g and the sea mussels, 7–8 g. The pooled specimens from the four kinds of tissues from the whelks weighed about 2 g each, and those from the sea mussels 0.7 and 2.3 g each (see Table 1).

In the **starfish** only the pyloric caeca were studied. About 2.4 g was obtained from two large specimens (body weights 178 and 205 g; 30 cm in diameter), as further specified in Table 1.

Only rather small specimens of the **sea-squirt** could be obtained at the time of the year when the preparations were made. For obvious reasons, the body weight and length could only be roughly estimated (2–3 g and 4–6 cm, respectively). The oesophagus, the stomach, and the gut were dissected from about 40 sea-squirts.

^{*} The samples from amphibians and reptiles were immediately extracted in acid ethanol (see below) without previous freezing.

The weights of the pooled samples were 1–2.5 g for the stomach and gut, whereas the minced material from the short oesophagus specimens only weighed 64 mg altogether (Table 1).

The supply of **lancelots** was limited to 11 individuals with a body length varying from 3 to 5 cm and a body weight of 0.05 to 0.35 g. Specimens for histological and immunocytochemical investigations were taken from three levels of the gut, tentatively called the midgut, the hindgut, and the rectum. A fourth sample was taken from the liver caecum ("Leber-blindsack"). For the radioimmunological assays, it was impossible to split up the alimentary tract into separate regions. Even when all parts were pooled, the total amount of minced material was less than 60 mg (Table 1).

In the **hagfish**, extracts were made from two locations: (a) excised islet organs with adjacent bile ducts and (b) stripped mucosa from the whole intestinal tube. The islet specimens and those from the gut mucosa were taken from hagfish (130 and 20, respectively) of average size (body weights and lengths about 35–50 g and 30–35 cm, respectively), which had been fasting for about a week in large tanks, equipped with running sea-water of high salinity (about 3‰) at +6° to +8°C. About 0.5 g of islet-bile-duct parenchyma and more than 2 g of gut mucosa were obtained (Table 2). As recent comprehensive histological, histochemical, and immunocytochemical investigations have been made of the GEP endocrine system in this animal (see ÖSTBERG, 1976), only a few samples were taken from the islet organ, bile duct and gut mucosa for light microscopical studies.

As to **cartilaginous fish**, only two female spiny dogfish could be obtained. They were kept in large basins of the same kind as those used for the hagfish, and were fasting when sacrificed. Their body weights were 4.9 and 3.2 kg. Specimens were taken from the oesophagus, three levels of the stomach ("corpus," "canalis," and "pylorus" regions), the large gut with its spiral valves ("Spiralfalte"), the "rectal gland" ("Rektaldrüse"), and the compact pancreatic gland. The material from the ventral lobe of the pancreas was not segregated from that of the dorsal lobe. Samples, weighing between 0.5 and 3 g, were obtained for radioimmunological assays (Table 2).

The most comprehensive light-microscopical investigations were made in the **daddy sculpin**. In five fish, 2 males and 3 females, weighing 52 and 72 g (the males) as well as 108, 240 and 288 g (the females), the whole length of the digestive tract was measured, and compared to the body length. Specimens for light-microscopical studies were taken from 13 different levels: one from the lowest parts of oesophagus, two each from the cardiac region and the "corpus-canalıs" regions of the stomach, one from the pyloric appendages, six from different levels of the gut, and one from the anal region. In addition, some material was taken from the two principal islets ("Brockmann's corpuscles") to supplement previous light-microscopical studies of this kind of teleostean islet parenchyma (FALKMER, 1961; 1966, FALKMER and PATENT, 1972; FALKMER and ÖSTBERG, 1977). Samples for radioimmunological SRIF assays of the islet parenchyma were obtained by dissecting 56 daddy sculpin of either sex (slight predominance of females), with body weights varying from 60 to 390 g. Both fasting and non-fasting fish were used. The pyloric and splenic islets were not separated. In order to get an idea of the orders of magnitude of the methodological errors involved, the pool of the collected islets was divided into two parts, one weighing about 200 mg and the other about 250 mg, which afterwards were prepared in the same manner.

For the SRIF assays of the digestive tract, three kinds of minced mucosa were sampled, *viz* from the stomach, the pyloric appendages, and the gut. Five daddy sculpin were used for this purpose, four females, weighing from 125 to 170 g, and one male, 110 g. The samples obtained weighed between 1 and 3 g (Table 2).

Seven **frogs**, 3 males and 4 females, were caught and kept in captivity for several months. Except for one female, weighing 20 g, their body weights were about 30 g. The frogs were not fasting. They were killed by decapitation. Samples were taken from the antrum of the stomach, from the duodenum, and from the compact pancreas. The specimens were not pooled. Their weights varied from 15 to 65 mg.

Seven **snakes** were collected: five grass snakes and two adders. One grass snake was a female; all the others were males. The body lengths and weights varied from about 50 to 60 cm and 25 to 60 g, respectively, except for the female grass snake that was 88 cm long and weighed more than 140 g. In the grass snakes only the compact pancreatic glands were examined. In the two adders, however, samples were taken also from the stomach, the duodenum, and the ileum. As in the amphibia, the samples of pancreatic parenchyma and the gastrointestinal (GI) tract were not pooled. The weights of the individual samples varied from 0.04 to 0.26 g. In the adders the whole pancreatic gland was minced, whereas in the grass snakes only representative parts were taken.

Light-microscopical techniques

All the cut sections of the specimens taken for light microscopical investigation were stained (see FALKMER and ÖSTBERG, 1977) by: (a) the aldehydefuchsin-trichrome technique, mainly to visualize the insulin-producing B-cells; (b) Grimelius' silver nitrate procedure, mainly to detect glucagon-producing A-cells, as well as some other kinds of argyrophil endocrine cells in the GI tract (see SOLCIA et al., 1977); and (c) Hellerström-Hellman's modification of the Davenport silver staining technique in order to visualize the SRIF-storing D-cells (FALKMER and HELLMAN, 1961).

In a few selected sections, detection of the SRIF-storing cells by the peroxidase-anti-peroxidase (PAP) immunocytochemical technique was attempted. The antisera used for this purpose were raised in rabbits by injecting synthetic SRIF, conjugated to hemocyanine from the limpet (ELDE and PARSONS, 1975). The details of the technique and the control procedures have also been previously reported (PARSONS et al., 1976). In these investigations, some supplementary studies were also made to visualize the insulin- and glucagon-producing parenchymal cells using the PAP technique. In these studies, previously described antisera were used (ERLANDSEN et al., 1976; JOHNSON et al., 1976).

Radioimmunological assays

After weighing, the frozen samples taken for radioimmunological assays were transferred to ice-cold 70% ethanol, acidified by 0.18 N HCl to pH 2, and homogenized in a Potter-Elvehjem glass homogenizer, equipped with a cooling mantle. An alternative homogenizing procedure was applied for the samples from the frogs and snakes by an ultrasonication technique, using an "MSE ultrasonic disintegrator, PG 100" (Crawley, U. K.). The samples treated with the Potter-Elvehjem outfit were homogenized and stirred for up to a few hours and, afterwards, centrifuged at 12,000–20,000 *g* at about 0–4°C. The ultrasonication lasted for 10–20 sec and the samples

Table 1. Observations by conventional light microscopy of the occurrence of various kinds invertebrates, as wells as the actual contents of SRIF in the same tissues. contents have

Main groups	Species	Tissue specimen	Light-microscopical	
			Argyrophil cells	
			Grim. ¹⁾	H-H ²⁾
Protostomata				
Arthropoda	<i>Carcinus maenas</i>	Stomach	(+)	0
		Gut (÷ caecum)	0	0
		Caecum	0	0
		Hepatopancreas	0	0
Mollusca	<i>Buccinum undatum</i>	Stomach	0	0
		Gut	0	0
		Hepatopancreas	0	0
		Protein gland	0	0
	<i>Mytilus edulis</i>	Gut	0	0
		Hepatopancreas	0	0
Deuterostomata				
Echinodermata	<i>Asterias rubens</i>	Pyloric caeca	(+)	0
Tunicata	<i>Cioa intestinalis</i>	Oesophagus	+	(+)
		Stomach	(+)	(+)
		Gut	+	0
Cephalochordata	<i>Branchiostoma lanceolatum</i>	“Midgut”	0	0
		“Hindgut”	+	0
		”Rectum”	(+)	(+)
		“Leberblind-sack”	0	0

¹⁾ Grimelius' silver nitrate procedure.

²⁾ Hellerström-Hellman's modification of the Davenport technique.

³⁾ Inverted values.

were left overnight at 4°C before centrifugation. The supernatants were sucked off and kept deep-frozen until assay. When only minor amounts of tissue samples were available, the final dilutions could be rather large (Tables 1 and 2).

The radioimmunological SRIF assays were performed by a previously described technique (see PETERSSON et al., 1977), using antisera raised in guinea-pigs by injecting synthetic SRIF conjugated to bovine thyroglobulin. The results of cross-tests between the rabbit and guinea-pig antisera, used in this study, showed that there were no differences between these two antisera. Although radioimmunological assays of this kind do not present any proofs that positive results imply the actual presence of SRIF, we have, for the case of simplicity, called the substance found in this study "SRIF" quite aware of this possible source of error.

In addition, radioimmunological assays of insulin and glucagon were performed

of islet endocrine cells in the GEP endocrine system of some protostomian and deuterostomian. For comparison, data from some previous analogous studies of the insulin also been included

occurrence of:		Radioimmunoassayable contents of somatostatin:			Insulin contents ⁴⁾ (mU/g. w. w.)
B-cells		Wet weight (g) of tissue sample	Final dilution of extract ³⁾	Yield (ng/g. w. w.)	
0		2. 178	11. 5	0	N. t. ⁵⁾
(+)		1. 089	22. 2	0	0
0		1. 703	14. 5	0	0
(+)		1. 524	16. 1	0	14. 5
+		2. 472	10. 3	0	11. 9
+		1. 757	14. 5	0	4. 2
0		1. 999	12. 5	0	0
0		2. 102	14. 3	0	N. t. ⁵⁾
(+)		0. 962	19. 9	0	N. t. ⁵⁾
0		2. 278	11. 1	0	N. t. ⁵⁾
+		2. 371	10. 7	0	60. 0
+		0. 064	47. 8	129	
+		1. 108	19. 1	134	5. 7
0		2. 501	9. 0	0	
(+)					
(+)		0. 057	53. 7	0	N. t. ⁵⁾
+					
+					

⁴⁾ Quoted from previous studies (FALKMER and WILSON, 1967; DAVIDSON et al., 1971).

⁵⁾ N. t.: not tested. +: Unequivocal occurrence. (+): Results of possibly positive but somewhat equivocal nature. 0: Essentially negative results.

on some of the extracts from snake pancreas by techniques used in one of our laboratories (OLSSON et al., 1976). The insulin assays were based on the procedure described by HEDING (1972), using mouse crystalline insulin standards and kits from the Radiochemical Centre, Amersham, U. K. The glucagon assay technique was that of HEDING (1971), employing the kits provided by Novo A-S, Copenhagen, Denmark (pig glucagon standards).

Results

Invertebrates

The results obtained in the protostomian and deuterostomian invertebrates have been summarized in Table 1, where also, for comparison, some data from previous

Fig. 1. Medium-power photomicrographs of the stomach mucosa of the sea-squirt (**A**) and the spiny dogfish (**B**) after application of the Hellerström–Hellman modification of the Davenport procedure. Whereas in the mucosa of the cartilaginous fish several, distinctly argyrophil cells occur in the bottom of the crypts (arrows in Fig. 1 **B**)—but not in the glands (*GI*)—no really blackened cells were found in the mucosa of the tunicate. Here, only some slightly darker cells of open type (i.e. reaching from the basal membrane to the luminal surface) were observed. Nevertheless, radioimmunoassayable SRIF was found, not only in extracts of the shark mucosa but also in that of the sea-squirt (Tables 1 and 2). This illustrates that the conventional Hellerström–Hellman technique to detect SRIF-storing D cells does not give reliable results in tissues of tunicates. $\times 500$

studies of a similar kind with regard to the occurrence of insulin-producing cells have been included. There seems to be fairly good correlation between the light microscopical observations and the results of the radioimmunoassays. From Table 1, SRIF appears to be, phylogenetically, rather an old hormone. Nevertheless, when compared with insulin, it does not occur until fairly late in evolution, appearing first in the tunicates. However, no cells were found that were distinctly argyrophil by the Hellerström–Hellman procedure—those cells in the mucosa of the oesophagus and stomach of the sea-squirt that were classified as “possibly argyrophil” are shown in Fig. 1A. They are darker than the other cells and seem to have basal granules reaching all the way from the basal membrane to the lumen. Thus, they might be endo-

crine cells of open type. It could not be ascertained whether they were identical to the cells of open type that were argyrophil by Grimelius' procedure. We have not made any ultrastructural studies nor any immunocytochemical investigations of them to show whether they actually represent the production site of the SRIF.

Only a few cells of this kind were occasionally observed in the most posterior parts of the intestinal tube of the amphioxus. The sparse occurrence of these cells, and the high dilutions that had to be made, can explain why a minor production of SRIF might have escaped detection in this study. Supplementary immunocytochemical investigations using the PAP technique are currently being made.

Vertebrates

A summary of the observations made in the ectothermic vertebrates is shown in Table 2. It shows that SRIF occurs both in the islet parenchyma and in the gut mucosa of agnathan as well as gnathostomian species. The histological procedures used to detect the A and B cells gave the expected results, and, except for in the cyclostomes, it seems from the Table to be possible to trace the SRIF-storing cells by conventional light-microscopical procedures used to detect D cells (Fig. 1 B, 2).

It should be stressed, however, that the positive results given in Table 2 and Fig. 2 B often are the exceptions rather than the rule. Consequently, it was usually difficult, and even impossible, to visualize these D cells by the standard technique. Modifications often had to be made in the original Hellerström-Hellman variant of the Davenport procedure, for instance with regard to fixation and the impregnation technique, in order to demonstrate any real argyrophilia when the specimens to be analyzed were from the GEP endocrine system of most of these ectothermic vertebrates. This statement applies in particular to SRIF-containing GEP tissues of the daddy sculpin, the frogs (Fig. 2B), and the snakes. In the hagfish, no modifications whatsoever gave any positive results. In snake islets, the argyrophilia could only be demonstrated in specimens fixed in Zenker's fluid (Fig. 4 C). It was only in the shark that the argyrophilia of the D cells could easily be demonstrated in Bouin-fixed specimens (Fig. 1 B, 2 A). Despite all these shortcomings with the Hellerström-Hellman procedure, which are well-known from the literature (see FALKMER and PATENT, 1972; FALKMER and ÖSTBERG, 1977), and which might erroneously be interpreted as indicating the absence of D cells in large parts of the GEP endocrine system of ectothermic vertebrates, practically all the tissues examined were found by radioimmunoassay to contain SRIF, sometimes in appreciable quantities (Tables 2-5).

In contrast, the SRIF-storing D cells could easily be visualized in almost all of these tissues where SRIF was found by radioimmunoassay and where the Hellerström-Hellman procedure more or less failed—even in those of the islet organ of the hagfish—when the PAP technique was used (Fig. 3 B, 4 A). These immunocytochemical examinations are still not finished. In the hagfish gut mucosa, the SRIF-storing cells have still not been detected; some other cells in the GI tract of vertebrates also remain to be studied.

By the PAP technique, the D cells of the other parts of the **hagfish** GEP endocrine system were found to be mainly localized in the bile duct mucosa, whereas the number of D cells in the islet parenchyma was low. This is in full agreement with previous observations from one of our laboratories (ÖSTBERG, 1976; VAN NOORDEN et al., 1977; FALKMER and ÖSTBERG, 1977) and also with the results of our quantitative assays

Table 2. Observations by conventional light microscopy of the occurrence of various vertebrates, as well as the actual

Main groups	Species	Tissue specimen	Light-microscopical
			Argyrophil
			Grim. ¹⁾
Agnatha			
Cyclostomi	<i>Myxine glutinosa</i>	Gut	+
		Islet organ	0
Gnathostomata			
Chondrichthyes	<i>Squalus acanthias</i>	Oesophagus	0
		Stom.: "Corpus"	+
		Stom.: "Canalis"	+
		Stom.: "Pylorus"	(+)
		Gut	(+)
		"Rectal gland"	0
		Pancreas	+
Osteichthyes	<i>Cottus scorpius</i>	Stomach	+
		Pyloric append.	+
		Gut	+
		Islet organ	+
Amphibia	<i>Rana temporaria</i>	Stomach	(+)
		Duodenum	(+)
		Pancreas	+
Reptilia	<i>Vipera berus</i>	Stomach	+
		Duodenum	+
		Ileum	+
		Pancreas	+
	<i>Natrix natrix</i>	Pancreas	+

¹⁾ Grimelius' silver nitrate procedure.²⁾ Hellerström-Hellman's modification of the Davenport technique.³⁾ Inverted values.

(Tables 2 and 3). To deem from the latter (Table 3), the D cells appear to constitute only about 1% of the total endocrine parenchyma in the islet organ and bile duct mucosa, and this might well be the case (ÖSTBERG, 1976; VAN NOORDEN et al., 1977; FALKMER and ÖSTBERG, 1977).

In the **cartilaginous fish**, *Squalus acanthias*, the correspondence between the SRIF contents and the presence of parenchymal endocrine cells, giving distinct argyrophil reactions with the standard Hellerström-Hellman technique, was excellent (Table 2 and Figs. 1 B, 2 A). In the GI tract, the D cells seemed to be mainly of open type

kinds of islet endocrine cells in the GEP endocrine system of some ectothermic contents of SRIF in the same tissues

occurrence of:		Radioimmunoassayable contents of somatostatin:		
cells	B-cells	Wet weight (g) of tissue sample	Final dilution of extract ³⁾	Yield (ng/g. w. w.)
H-H ²⁾				
0		2. 325	10. 9	5
0	+	0. 461	7. 6	7, 900
0	0	2. 958	7. 8	0
+	0	2. 411	10. 5	926
+	0	0. 541	19. 6	1, 970
+	0	0. 365	22. 9	421
(+)	0	2. 702	9. 5	103
0	0	2. 520	9. 0	0
+	+	3. 074	7. 5	3, 800
0	0	2. 978	7. 5	0
0	0	1. 584	15. 5	0
0	0	1. 277	19. 0	0
		0. 208	14. 5	278, 000
+	+	0. 257	7. 9	146, 000
0	0	0. 046 ⁴⁾	40. 0	2, 300 ⁴⁾
0	0	0. 035 ⁴⁾	40. 0	4, 700 ⁴⁾
+	+	0. 030 ⁴⁾	40. 0	17, 100 ⁴⁾
+	0	0. 067 ⁴⁾	20. 0	1, 246 ⁴⁾
0	0	0. 125 ⁴⁾	20. 0	300 ⁴⁾
(+)	0	0. 053 ⁴⁾	20. 0	303 ⁴⁾
(+)	+	0. 159 ⁴⁾	20. 0	25, 700 ⁴⁾
(+)	+	0. 126 ⁴⁾	20. 0	18, 000 ⁴⁾

⁴⁾ Mean values. +: Unequivocal occurrence.

(+): Results of possibly positive but somewhat equivocal nature.

0: Essentially negative results.

(FUJITA, 1976). Here, however, ultrastructural studies remain to be performed to verify this assumption. The D cells were mainly localized at the bottom of the crypts and not in the glands (Fig. 1 B). In the duct-associated islets, the D cells were only rarely observed in the duct epithelium (Fig. 2 A).

The two giant principal islets of the **daddy sculpin** (see FALKMER et al., 1975) showed the highest contents of all the tissues investigated (Table 2). The conspicuous difference between the duplicate samples shows, however, that the methodological errors of the homogenization and radioimmunoassay procedures were rather large

Fig. 2. Medium-power photomicrographs of the islets of Langerhans in the spiny dogfish (A) and in a frog (B), showing the rather frequent occurrence of argyrophil D cells (black) as visualized by the Hellerström–Hellman technique in these two kinds of rather primitive gnathostomian vertebrate islet parenchyma. In both, there is still a close association between the islet cells and the duct epithelium (*Dc*), and the shape of the islets is irregular (*Ex*=exocrine, acinar pancreatic parenchyma). Both the chondrichthyeian and the amphibian pancreatic glands also contained large amounts of SRIF (Tables 2 and 4). Nevertheless, in the frogs used in this study, it was most difficult to find any argyrophil cells (like those shown in this photomicrograph) by the routine Hellerström–Hellman method; again showing its shortcomings. $\times 500$

(Table 2). Still, the sculpin islet parenchyma contained about 10 times more than any other pancreatic or islet tissue. This is easily understood from the morphology of the sculpin principal islets (FALKMER, 1961; 1966; FALKMER and HELLMAN, 1961; FALKMER and ÖSTBERG, 1977), where previous research has demonstrated that the D cells occupy the central region together with the B cells. This has now been confirmed by the PAP technique (Fig. 3), which showed that the glucagon-producing cells are located in the peripheral region and that those responsible for the insulin and SRIF production are limited to the central region. In the sculpin islet parenchyma there is, consequently, a close topographical relationship between the B and D cells. A quantitative comparison between the contents of insulin and SRIF in the sculpin islet parenchyma reveals that about 5 times more insulin is produced (Table

Table 3. The radioimmunoassayable SRIF contents in the islet organ of a cyclostome and that of a bony fish (i.e. the Brockmann corpuscles) in comparison with the actual contents of insulin in the same two kinds of isolated islet parenchyma

Subclass	Species	SRIF ¹⁾ (ng/mg w. w.)	Insulin ²⁾ (ng/mg w. w.)
Cyclostomi (Myxinoidei)	<i>Myxine glutinosa</i> (Atlantic hagfish)	8	1,000
Osteichthyes (Teleostei)	<i>Cottus scorpius</i> (Daddy sculpin)	212	1,040

¹⁾ cf. Table 2.

²⁾ Yields after purification to monocomponent hormones (PETERSON et al., 1975; EMDIN and FALKMER, unpublished).

Table 4. Individual variations in the SRIF contents of three parts of the GEP endocrine system in the frog, *Rana temporaria*

Body weight (g)	Contents of somatostatin (ng/mg wet weight)		
	Stomach	Duodenum	Pancreas
30	5.2	5.4	20.4
30	5.9	4.4	16.6
30	0.7	2.6	14.2
30	0.8	4.1	13.5
20	0.7	8.3	31.6
27	0.8	1.9	8.9
33	1.8	6.3	14.3
Mean $\pm$ S.D.	2.3 $\pm$ 2.3	4.7 $\pm$ 2.2	17.1 $\pm$ 7.3

Table 5. Comparisons between the radioimmunoassayable contents (in ng/mg wet weight) of somatostatin, glucagon, and insulin in a reptilian pancreatic gland. The relative amounts of the three hormones have been compared with available data from the literature (cf. FALKMER and ÖSTBERG, 1977) on the relative frequencies of D, A, and B cells in the islets of Langerhans in reptiles as found by conventional light microscopy and transmission electron microscopy

Species	Body weight (g)	Sex	SRIF	Glucagon	Insulin
Grass snake (<i>Natrix naotrix</i>)	63	Male	8	n. t. ¹⁾	49
	21	"	37	95	84
	45	"	23	68	132
	27	"	10	50	48
	143	Female	14	29	89
Means:			18	61	80
Crude ratios:			12%	38%	50%
Crude cell frequencies:			10% ²⁾	40% ³⁾	50% ⁴⁾

¹⁾ n. t. = not tested, ²⁾ D cells, ³⁾ A cells, ⁴⁾ B cells.

Fig. 3. Medium-power photomicrographs of parts of the principal islet (Brockmann corpuscle) situated close to the pyloric region of the daddy sculpin (*Cottus scorpius*). The sections have been immunocytochemically analyzed by the PAP procedure for the presence of glucagon-producing A cells (**A**) and SRIF-storing D cells (**B**). Both cell types occur in large amounts. The A cells (black in Fig. 3 **A**) occupy most of the peripheral region (*Per*) close to the connective-tissue capsule (*Cap*) of these giant islet bodies, whereas the D cells (black in Fig. 3 **B**) are located in the central region (*Cen*), together with the insulin-producing B cells (unstained, greyish). By the Grimelius' procedure the A cells are easily visualized, but it is only rare that the argyrophilia of the D cells can be demonstrated in bony fish islets by the Hellerström–Hellman technique despite the large SRIF contents found by radioimmunoassay (Tables 2 and 3).

A: $\times 500$, **B:** $\times 430$

3). As in the hagfish islet-bile-duct parenchyma, this interrelationship was found to reflect approximately the relative frequencies of B and D cells in the sculpin principal islets. The apparent complete absence of SRIF-storing cells in the sculpin GI tract was a rather surprising observation, and it will be further studied, i.e. by the PAP technique.

Rather large individual variations also were observed in the **amphibians** in the SRIF contents of the three parts of the GEP endocrine system investigated. This is best illustrated by the magnitude of the standard deviations in Table 4. Whether these variations are mainly of a technical-methodological or of a biological (different

states of nutrition) nature is difficult to determine. In any case, it is clear from Table 4 that there are no obvious co-variations of the SRIF contents of the three parts of the amphibian GEP endocrine system, and no relationship between the body weights and the SRIF contents. The cell responsible for the SRIF production remain to be studied by the PAP technique and by transmission electron microscopy, since, as mentioned above, the histological procedures we used were less successful than expected. Trials to relate the immunoassayable contents of SRIF to those of insulin in

Fig. 4. Medium-power photomicrographs of an islet of Langerhans in the grass snake, stained for argyrophil D cells by the Hellerström-Hellman technique (C), and analyzed by the PAP procedure for SRIF-storing D cells (A), glucagon-producing A cells (B), and insulin-producing B cells (D). The argyrophilia of the D cells (black in Fig. 4 C) could not be demonstrated in "Bouin-fixed" specimens, despite the fact that the cells could easily be visualized by the PAP technique. It was only after fixation in Zenker's fluid that pictures like those in Figure 4 C could be obtained. In the reptilian islets the D cells are preferentially located in the periphery (Fig. 4 A and C) close to the exocrine pancreatic parenchyma (*Ex* in Fig. 4 C). As in the bony fish (Fig. 3) the B cells (Fig. 4 D) occur together with the D cells. In contrast to the teleostean islets, however, the A cells are mainly located in the central parts of the islets (Fig. 4 B). $\times 400$

amphibian pancreas failed to give any relevant results, probably due to deficient cross-reactivities between frog insulin and the mouse insulin antibodies we used in our assay procedure.

In the **snakes**, however, there seemed to be no such species-specificity problems with the radioimmunoassay procedures. As shown in Table 5, the rough ratios of the average contents of SRIF, glucagon, and insulin in this kind of reptilian pancreas were found to correspond surprisingly well with the relative frequencies of the D, A, and B cells in reptilian islets of Langerhans, as reported in the literature (see FALKMER and ÖSTBERG, 1977).

As these results are based on only 4–5 assays with large individual variations (Table 5), this correlation could, of course, be fortuitous, awaiting confirmation by more comprehensive and systematic studies. There seemed to be no relationship of the SRIF (or glucagon, or insulin) contents to the sex or body size of the snakes.

When the topographical distribution of the SRIF-storing cells was investigated by means of the PAP technique, it was found that there was a tendency for the D cells to occur in the periphery (Fig. 4 A, C) together with the B cells (Fig. 4 D), whereas the A cells (Fig. 4 B) mainly occupied a central position in the islets. The regional distribution was, however, not at all as distinct as in the sculpin principal islets (Fig. 3).

Discussion

As suggested previously in reviews on the comparative endocrinology of the GEP endocrine system (FALKMER and PATENT, 1972; FALKMER and ÖSTBERG, 1977), it seems as if all the islet cells originate from the GI tract mucosa. During evolution the insulin-producing B cells first leave the gut and bud out from the bile duct mucosa to form a separate islet organ. The B cells are apparently followed by the SRIF-storing D cells, whereas the glucagon-producing A cells are the last to enter the islet parenchyma. Some support for such a working hypothesis is obtained from the results of the present investigation. They indicate that in the GEP endocrine system SRIF is, phylogenetically, rather an old hormone, occurring not only in all vertebrates but also in some deuterostomian invertebrates. However, although the D cells follow the B cells in the formation of the first islet organ in evolution (FALKMER and ÖSTBERG, 1977), they differ from the B cells by still remaining to some extent in the GI mucosa as well; a feature that they share with the A cells at later stages in evolution.

In several of the animals included in our study, a topographical interrelationship was observed between the D and B cells, e.g. in the sea-squirt, the hagfish, the daddy sculpin, and the snakes. Whether this was also the case in the shark and the frog islets will be further studied in the future. If this B–D cell interrelationship is found to be a general phenomenon in deuterostomian invertebrates and lower vertebrates, this might give some clues as to the evolutionary aspects of the functional subdivision of the islets of Langerhans (see ORCI and UNGER, 1975; HELMSTAEDTER et al., 1976).

The marked discrepancy between the results of the immunocytochemical PAP procedure and the conventional Hellerström–Hellman technique to visualize the D cells light-microscopically in all these ectothermic vertebrates, except for in the shark, definitely limits the usefulness of the latter procedure for use in comparative studies of the GEP endocrine system. This is a pity since it would have been valuable to have access also to a non-immunological test procedure to detect SRIF-storing

cells. Thus, the present results may be minimum figures only. The positive results obtained, so far, with the radioimmunoassays and the PAP technique indicate, however, that no marked species-specificity occurs in this rather small peptide hormone, and that the concentrations observed may be correct.

When the Hellerström–Hellman technique works adequately, as in cartilaginous fish and in snakes after some modifications, it gives nice cytological results, allowing minute cytological details of the D cells to be studied in the sections. Thus, it seems by this technique (Fig. 2) that the D cells in the GI tract are mainly of open type (FUJITA, 1976), possibly showing some evolutionary aspects on the recent finding in cats that SRIF can be released into the lumen of the antrum of stomach (UVNÄS–WALLENSTEN et al., 1977). Our observations with the Grimelius' and Hellerström–Hellman techniques in the sea-squirt also agree with those of the recent report by FRITSCH (1976). Excellent agreement also exists with regard to our observations with the PAP procedure in the daddy sculpin with those of JOHNSON and associates (1976) and in the hagfish with those of VAN NOORDEN and associates (1977), as mentioned previously.

The results of the quantitative assays also agreed well with those available in the literature. The order of magnitude was the same for the SRIF values of the three hagfish briefly reported by VALE and associates (1976) and the crude ratios between the three islet hormones in the snake pancreas (Table 5) were in some respects similar to those reported for the pancreas in (from an evolutionary point of view) closely related birds (WEIR et al., 1976). In the splenic lobe of the avian pancreas which is known to be particularly rich in islet parenchyma (cf. FALKMER and ÖSTBERG, 1977), the SRIF content was reported to be 23 ng/mg (WEIR et al., 1976) which is similar to the values we observed in snakes. The insulin and, in particular, the glucagon values were higher, however, reaching up to 600 ng/mg wet weight (WEIR et al., 1976). Still, it seems as if the principal islets in the teleost fish, *Cottus scorpius*, are the gross anatomic structures that contain the largest amounts of SRIF in the Animal Kingdom, about 200 ng/mg wet weight.

References

- Davidson, J. K., S. Falkmer, B. K. Mehrotra and S. Wilson:** Insulin assays and light microscopical studies of digestive organs in protostomian and deuterostomian species and in coelenterates. *Gen. comp. Endocrinol.* 17: 388–401 (1971).
- Dubois, M. P., J. Barry and J. Leonardelli:** Mise en évidence par immunofluorescence et répartition de la somatostatine (SRIF) dans l'éminence médiane des Vertébrés (Mammifères, Oiseaux, Amphibiens, Poissons). *C. r. Acad. Sci. (Paris)* 279: 1899–1902 (1974).
- Elde, R. P. and J. A. Parsons:** Immunocytochemical localization of somatostatin in cell bodies of the rat hypothalamus. *Amer. J. Anat.* 144: 541–548 (1975).
- Erlandsen, S. L., O. D. Hegre, J. A. Parsons, R. C. McEvoy and R. P. Elde:** Pancreatic islet cell hormones distribution of cell types in the islet and evidence for the presence of somatostatin and gastrin within the D cell. *J. Histochem. Cytochem.* 24: 883–897 (1976).
- Falkmer, S.:** Experimental diabetes research in fish. On the morphology and physiology of the endocrine pancreatic tissue of the marine teleost, *Cottus scorpius*, with special reference to the role of glutathione in the mechanism of alloxan diabetes using a modified nitroprusside

- method. *Acta endocrinol.* 37 (Suppl. 59): 1-122 (1961).
- Falkmer, S.:** Quelques aspects comparatifs des cellules A pancréatiques et du glucagon. *Ann. Endocrinol.* 27: 321-330 (1966).
- Falkmer, S., J. F. Cutfield, S. M. Cutfield, G. G. Dodson, J. Gliemann, S. Gammeltoft, M. Marques, J. D. Peterson, D. F. Steiner, F. Sundby, S. O. Emdin, N. Havu, Y. Östberg and L. Winbladh:** Comparative endocrinology of insulin and glucagon production. *Amer. Zool.* 15 (Suppl. 1): 255-270 (1975).
- Falkmer, S. and B. Hellman:** Identification of the cells in the endocrine pancreatic tissue of the marine teleost *Cottus scorpius* by some silver impregnation procedures. *Acta morphol. neerland-scand.* 4: 145-152 (1961).
- Falkmer, S. and Y. Östberg:** Comparative morphology of pancreatic islets in animals. In: (ed. by) B. W. Volk and K. F. Wellman: *The diabetic pancreas*. Plenum Publ., New York, 1977. (p. 15-60).
- Falkmer, S. and G. J. Patent:** Comparative and embryological aspects of the pancreatic islets. In: (ed. by) D. F. Steiner and N. Freinkel: *Handbook of physiology*. Sect. 7. Vol. I. *The endocrine pancreas*. Williams and Wilkins Co., Baltimore, 1972. (p. 1-23).
- Falkmer, S. and S. Wilson:** Comparative aspects of the immunology and biology of insulin. *Diabetologia* 3: 519-528 (1967).
- Fritsch, H. A. R.:** The occurrence of argyrophilic and argentaffin cells in the gut of *Ciona intestinalis* L. *Cell Tiss. Res.* 175: 131-135 (1976).
- Fujita, T.:** The gastro-enteric endocrine cell and its paraneuronic nature. In: (ed. by) R. E. Coupland and T. Fujita: *Chromaffin, enterochromaffin and related cells*. Elsevier, Amsterdam, 1976. (p. 204-208).
- Heding, L. G.:** Radioimmunological determination of pancreatic and gut glucagon in plasma. *Diabetologia* 7: 10-19 (1971).
- : Determination of total serum insulin (IRI) in insulin-treated diabetic patients. *Diabetologia* 8: 260-266 (1972).
- Helmstaedter, V., G. E. Feurle and W. G. Forssmann:** Insulin-, glucagon-, and somatostatin-immunoreactive endocrine cells in the equine pancreas. *Cell Tiss. Res.* 172: 447-454 (1976).
- Hökfelt, T., S. Efendić, C. Hellerström, O. Johansson, R. Luft and A. Arimura:** Cellular localization of somatostatin in endocrine-like cells and neurons of the rat with special references to the A₁-cells of the pancreatic islets and to the hypothalamus. *Acta endocrinol.* 80 (Suppl. 200): 1-41 (1975).
- Johnson, D. A., J. L. Torrence, R. P. Elde, G. E. Bauer, B. D. Noe and D. J. Fletcher:** Immunohistochemical localization of somatostatin, insulin and glucagon in the principal islets of the anglerfish (*Lophius americanus*) and the channel catfish (*Ictalurus punctata*). *Amer. J. Anat.* 147: 119-124 (1976).
- Noe, B. D., G. C. Weir and G. E. Bauer:** Somatostatin biosynthesis in anglerfish islets. *Endocrinology* (1978, in press).
- Olsson, S.-E., A. Andersson, B. Petersson and C. Hellerström:** Effects of somatostatin on the biosynthesis and release of insulin from isolated pancreatic islets. *Diabète Metab.* 2: 199-202 (1976).
- Orci, L. and R. Unger:** Functional subdivision of islets of Langerhans and possible role of D-cells. *Lancet*: 1233-1244 (1975).
- Östberg, Y.:** The entero-insular endocrine organ in a cyclostome, *Myxine glutinosa*. A light and fluorescence microscopical, histochemical and ultrastructural study, aiming to give some evolutionary aspects on the origin of islet parenchymal cells and the entero-insular axis. *Umeå Univ. Med. Diss.* 15: 1-41 (1976).
- Parsons, J. A., S. L. Erlandsen, O. D. Hegre, R. C. McEvoy and R. P. Elde:** Central and peripheral localization of somatostatin. Immunoenzyme immunocytochemical studies. *J. Histochem.* 24: 872-882 (1976).

- Pearse, A. G. E., J. M. Polak and S. R. Bloom:** The newer gut hormones. Cellular sources, physiology, pathology, and clinical aspects. *Gastroenterology* 72: 746-761 (1977).
- Peterson, J. D., D. F. Steiner, S. O. Emdin and S. Falkmer:** The amino acid sequence of the insulin from a primitive vertebrate, the Atlantic haffish (*Myxine glutinosa*). *J. biol. Chem.* 250: 5183-5191 (1975).
- Petersson, B., R. P. Elde, S. Efendić, T. Hökfelt, O. Johansson, R. Luft, E. Cerasi and C. Hellerström:** Somatostatin in the pancreas, stomach, and hypothalamus of the diabetic Chinese hamster. *Diabetologia* 13: 463-466 (1977).
- Solcia, E., J. M. Polak, L. I. Larsson, F. Sundler, J. Lechago, L. Grimelius, T. Fujita, W. Creutzfeldt, W. Gepts, S. Falkmer, G. Lefranc, P. Heitz, C. Bordi, E. Hage, A. M. J. Buchan, S. R. Bloom and M. I. Grossman:** Lausanne 1977 classification of gastroenteropancreatic (GEP) endocrine cells. In: (ed. by) S. R. Bloom: Gut hormones. *Proc. Int. Symp., Lausanne, June 18-19, 1977.* Churchill Livingstone, Edinburgh, 1977 (in press).
- Uvnäs-Wallensten, K., S. Efendić and R. Luft:** Release of somatostatin into the antral lumen of cats. *Acta physiol. scand.* 99: 126-128 (1977).
- Vale, W., P. Brazeau, C. Rivier, M. Brown, B. Boss, J. Rivier, R. Burgus, N. Ling and R. Guillemin:** Somatostatin. *Rec. Prog. Horm. Res.* 31: 365-397 (1975).
- Vale, W., N. Ling, J. Rivier, J. Villarreal, C. Rivier, C. Douglas and M. Brown:** Anatomic and phylogenetic distribution of somatostatin. *Metabolism* 25 (Suppl. 1): 1491-1494 (1976).
- Van Noorden, S., Y. Östberg and A. G. E. Pearse:** Localization of somatostatin-like immunoreactivity in the pancreatic islets of the hagfish, *Myxine glutinosa*, and the lamprey, *Lampetra fluviatilis*. *Cell Tiss. Res.* 177: 281-285 (1977).
- Weir, G. C., P. C. Goltsos, E. P. Steinberg, E. P. and Y. C. Patel:** High concentration of somatostatin immunoreactivity in chicken pancreas. *Diabetologia* 12: 129-132 (1976).
- Wilson, W. and S. Falkmer:** Starfish insulin. *Can. J. Biochem.* 43: 1615-1624 (1965).

Professor Sture FALKMER
Institute of Pathology
S-901 87 Umeå 6
Sweden