MEASUREMENT AND PARTIAL CHARACTERIZATION OF SOMATOSTATIN-LIKE MATERIAL IN THE CENTRAL NERVOUS SYSTEM

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SOMATOSTATIN IN THE CENTRAL NERVOUS SYSTEM

ABSTRACT

A radioimmunoassay was developed to determine the concentration of immunoreactive somatostatin (IRS) in crude and partially-purifed extracts of various anatomical regions of the nervous system, including the retina. Affinity chromatography using somatostatin antiserum served to concentrate IRS from the retina and other brain regions. Retinal IRS from rats and humans inhibited the release of growth hormone in a bioassay in a similar manner as did synthetic somatostatin. Affinity chromatography-purified extracts of rat median eminence, anterior hypothalamic-preoptic area, amygdala and cerebral cortex, when subjected to gel filtration chromatography, eluted with similar profiles characterized by four IRS peaks of which the third to elute was largest and had a coincidental elution position with synthetic somatostatin. The IRS in each peak from all four brain regions was biologically active as assessed by its ability to inhibit growth hormone secretion.

RESUME

On a fait un dosage radio-immunologique pour déterminer la concentration de la somatostatine immunoréactive (SIR) dans des extraits tissulaires bruts et partiellement purifiés provenant des différentes régions anatomiques du système nerveux, y compris de la rétine. La chromatographie d'affinité avec usage d'antisérum de somatostatine a servi à concentrer la SIR de la rétine et des autres régions du cerveau. La SIR rétinienne de rats et d'hommes a inhibé la libération de l'hormone de croissance dans un dosage biologique, d'une façon comparable à celle de la somatostatine synthétique. Des extraits purifiés par chromatographie d'affinité et provenant de l'éminence médiane, de la zone préoptique antérieure de l'hypothalamus, d'amygdales et du cortex cérébral de rats, lorsqu'on les a soumis à la chromatographie de filtration par gel, ont été élués avec des profils similaires caractérisés par quatre pics de SIR, dont le troisième, le plus grand, présentait une position d'élution coïncidant avec la somatostatine synthétique. La SIR dans chacun des pics des quatres régions du cerveau était biologiquement active, comme l'a prouvé son pouvoir d'inhiber la sécrétion de l'hormone de croissance.

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ABBREVIATIONS

ACh	acetylcholine
ACTH	adrenocorticotropic hormone
APUD	amine precursor uptake and decarboxylation
BSA	bovine serum albumin
CNS	central nervous system
CSF	cerebrospinal fluid
cpm	counts per minute
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine-3'-5',-monophosphate
db cAMP	dibutyryl cyclic adenosine monophosphate
DNAse	deoxyribonuclease
EDTA	ethylene diamine tetraacetic acid
FSH	follicle stimulating hormone
GH	growth hormone
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSA	human serum albumin
IRS	immunoreactive somatostatin
icv	intracerebral ventricular
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
ME	median eminence
OVLT	organum vasculosum of the lamina terminalis
PETH	pink-eyed, tan-hooded (rats)
PRL	prolactin
RIA	radioimmunoassay
REM	rapid eye movement (sleep)
SWS	slow wave sleep
SS-AS	somatostatin antiserum
SME	stalk median eminence
SD	standard deviation
SEM	standard error of the mean
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone

INTRODUCTION

I. GENERAL OBSERVATIONS ON PEPTIDES COMMON TO THE BRAIN AND GASTROINTESTINAL TRACT

Because the studies described herein almost solely concern the peptide somatostatin as it exists in the nervous system it would be appropriate as an introduction to consider somatostatin in a more general sense as a member of a conceptually new class of biologically active peptides which are localized in the nervous system and gastrointestinal tract or pancreas. Von Euler and Gaddum (1) in 1931 first identified biological activity due to substance P in extracts of horse brain and intestine, but it was not until 1970-71 that the peptide responsible for the biological activity was isolated from brain tissue of cattle and its structure was characterized (2,3). A number of radioimmunoassay (RIA) and immunohistochemical studies in 1975 demonstrated somatostatin-like activity distributed widely throughout the nervous system, pancreas, and gastrointestinal tract. Subsequently the list of peptides common to the brain, pancreas and/or gastrointestinal tract has grown to include the several indicated in Table 1. Of this list, only somatostatin, substance P, and neurotensin have been isolated from both brain and gastrointestinal tract or pancreas and have had their amino acid sequences determined. Evidence for the inclusion of the others in the group of brain-gut peptides is based on the demonstration of immunological reactivity, either by RIA or immunohistochemistry, and is therefore suggestive, but not definitive. For instance, controversy exists with respect to whether thyrotropin releasing hormone (TRH)-like immunoreactivity in a variety of extrahypothalamic tissues and fluids represents true TRH or an immunologically cross-reactive non-TRH material (41-44).

These peptides and in particular somatostatin have shown a wide variety of biological actions in many experimental models that when considered along with the anatomical localization of the peptides have revitalized the concept of paracrine secretion (45). This term refers to the release from cells of a chemical transmitter into the interstitial space to influence the function of adjacent or nearby cells. Although classical neurotransmission

Table 1. Peptides Identified From Brain, Pancreas or Gut

S=amino acid sequence determined on isolated peptide

I=identification primarily by immunological methods

Peptide	Brain	Gut	Pancreas	References
Somatostatin	S	I	S	4-7
Substance P	S	S		2,3,8
Neurotensin	S	S		9,10
Adrenocorticotropin (ACTH)	I	I		11-13
Bombesin	I	I		14,15
Cholecystokinin	I	S		16,17
β-endorphin	I		I	18,19
Enkephalins	S	I		20,21
Gastrin	I	S		22
Glucagon	I	I	S	23
Insulin	I		S	24
Luteinizing hormone releasing hormone (LHRH)	S		I	25-27
Motilin	I	S		28
Pancreatic Polypeptide	I	I	S	29-31
Thyrotropin Releasing hormone (TRH)	S	I	I	32-37
Vasoactive intestinal polypeptide (VIP)	I	S		38-40

would strictly fit the description of paracrine secretion the term is most commonly used in reference to the peptides and cells of the pancreatic islets and gastrointestinal glands. Feyrter (46) in 1938 first brought attention to the common histological characteristics of a series of argentaffin "clear cells" in the gastrointestinal tract in a paper titled *Uber Diffuse Endokrine Epitheliale organe* suggesting the functional role of these cells. He subsesequently referred to the series of "clear cells" as constituting a *parakinen* system to emphasize his hypothesis that the cells acted on their neighbours (47). The evidence to support a paracrine function for somatostatin will be detailed below, but in summary the argument for paracrine secretion remains presumptive based primarily on anatomical studies and the effects of exogenously administered peptides.

The APUD (amine precursor uptake and decarboxylation) concept of Pearse bears discussion here because it receives considerable support by the existence of the brain-gut peptides (48-50). The original formulation of the APUD concept in 1966 referred to a group of endocrine cells which shared cytochemical characteristics of which the most prominent was the production of biogenic amines (51). Originally, Pearse suggested these cells were derived from embryological precursor cells of a "neural origin, perhaps coming from the neural crest" (50). Subsequent embryological developmental experiments, including particularly those of LeDouarin (52) using the technique of allografting quail embryonic neural tissue into chick embryos to produce quail-chick chimeras, demonstrated that only 6 or 7 out of 40 APUD cells actually arose from the neural crest. In a recent formulation a second subgroup of APUD cells, including the hypothalamus, pituitary and parathyroid glands, was proposed to originate from neuroectoderm or specialized ectoderm such as the placodes (50,53). The peptide-containing cells of the gastrointestinal tract and pancreas are of obscure embryological origin, as evidenced by Pearse's suggestion that they arise from the primitive ectoblast (49,50).

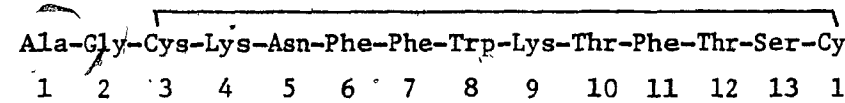
Although the APUD hypothesis has been of great importance by conceptually linking diverse cell types, perhaps an inordinate amount of attention in the literature has been given to attempts to identify a common embryological origin of these cells. It appears to the author most clear to simply consider that several cells, including those of the brain, share a fundamental common

characteristic of expression of genes for synthesis of certain biologically active peptides and/or monoamines. The significance of this fact in terms of the regulation of biosynthesis and the expression of biological activity of the brain-gut peptides in different tissues presents an intriguing subject for future investigation.

II. SOMATOSTATIN

A. HISTORY

The discovery of somatostatin was a direct consequence of the studies of Guillemin and colleagues directed toward isolation and characterization of the hypothalamic factors that regulate the secretion of anterior pituitary hormones. This group and Schally and coworkers had previously validated the hypothalamic releasing hormone concept by their characterization of TRH (32,33) and luteinizing hormone releasing hormone (LHRH) (25, 26). During their search for growth hormone releasing factor* Guillemin and colleagues observed the presence of a growth hormone (GH) release inhibiting property in extracts of sheep hypothalami. They accomplished the isolation and determination of the amino acid sequence of a tetradecapeptide,



named somatostatin, or somatotropin release inhibiting factor, which accounted for the observed biological activity (4,55-57). A crucial factor in the successful isolation of somatostatin was the use of a highly sensitive and reproducible bioassay of inhibition of release of GH from dispersed anterior pituitary cells in culture (58). Krulich and colleagues (59-62) had previously observed a GH release inhibiting activity in acetic acid extracts of sheep and rat hypothalami and had described an anatomical localization of this inhibitory factor in the anterior hypothalamus and

*The reader interested in the history of research on growth hormone releasing factor, which is still uncharacterized, is referred to the comprehensive review by Reichlin (54).

median eminence (ME) of the rat which is basically similar to that subsequently determined for somatostatin by use of RIA and immunohistochemical methods.

Somatostatin has now additionally been isolated from pig hypothalamus (5), pigeon pancreas (6) and anglerfish pancreas (7). It has had the identical amino acid sequence as sheep hypothalamic somatostatin in all cases. Chemical synthesis of somatostatin (63-67) followed soon after its original isolation. Several subsequent studies using synthetic somatostatin and various experimental models unequivocally confirmed the inhibitory effect of somatostatin on the secretion of GH by the pituitary.

The historically important discoveries that extended the relevance of somatostatin beyond the confines of the hypothalamus and pituitary can be summarized as: 1. Somatostatin is localized widely throughout the nervous system, pancreas, and gastrointestinal tract, 2. Somatostatin reduces plasma levels of glucose and several peptide hormones, including insulin and glucagon, 3. Somatostatin affects nervous as well as pituitary function.

B. ASSAY METHODS

1. Radioimmunoassay (RIA)

Arimura *et al* (68) and Patel and colleagues (69) in 1975 reported the first RIAs for somatostatin using antisera raised against synthetic somatostatin, as has been done for all subsequent RIAs. Although these two studies were the only reports of somatostatin RIAs available when the work described in this thesis was begun, several other RIAs have been described in recent years (Table 2). All antisera included in table 2, except that of Gerich *et al* (82), were raised in rabbits, although sheep (82,85) and guinea pigs (86) have also generated antisera. Most investigators have used an immunogen of somatostatin covalently conjugated to a variety of larger molecules including human serum α globulin, thyroglobulin, bovine serum albumin, human serum albumin, and whelk or keyhole limpet hemocyanin. In addition, Epelbaum and colleagues (73) have produced an antiserum to somatostatin by a method involving immunization with a mixture of methylated bovine serum albumin and somatostatin that are not covalently conjugated. Because somatostatin contains no amino acid residue appropriate for labelling with radioactive iodine the analogs [Tyr^1] somatostatin, [Tyr^8] somatostatin, [Tyr^{11}] somatostatin, and N-Tyr-somatostatin have been used for this purpose

Table 2. Radioimmunoassay of Somatostatin

Reference	First author and year	Protein conjugated to somatostatin for immunization	Somatostatin analog labelled	Iodination reaction
68,70	Arimura '75	human serum α globulin	$[Tyr^{1}]$-SS N-Tyr-SS	lactoperoxidase
71	Kronheim, '76	whelk hemocyanin	$[Tyr^{1}]$-SS	chloramine T
72	Vale, '76	human serum albumin	$[Tyr^{11}]$-SS	chloramine T
73	Epelbaum, '77	(noncovalent)	$[Tyr^{1}]$-SS	lactoperoxidase
74	Makino, '77	human serum α globulin	N-Tyr-SS	lactoperoxidase
75	Diel, '77	bovine serum albumin	$[Tyr^{1}]$-SS	several assessed
76	Patel, '78	bovine thyroglobulin	$[Tyr^{1}]$-SS	chloramine T
77	Chayvialle, '78	bovine serum albumin	$[Tyr^{1}]$-SS	chloramine T
78	McIntosh, '78	bovine serum albumin	$[Tyr^{1}]$-SS	chloramine T
79	Chiba, '78	bovine serum albumin	N-Tyr-SS	chloramine T
80	Penman, '79	keyhole limpet hemocyanin	$[Tyr^{11}]$-SS	lactoperoxidase
81	Dupont, '79	bovine thyroglobulin	N-Tyr-SS	lactoperoxidase
82	Gerich, '79	whelk hemocyanin	$[Tyr^{11}]$-SS	chloramine T
83	Kumasaka '79	bovine serum albumin	$[Tyr^{8}]$-SS	lactoperoxidase
84	Gillioz '79	bovine serum albumin	N-Tyr-SS	lactoperoxidase

Table 2. Radioimmunoassay of Somatostatin (Con't)

Reference	Purification of labelled analog	Separation of bound and free label	Minimum detectable concentration, pg somatostatin	Site on somatostatin molecule that antisera bind	Dilution of antibody used
68,70	cation exchange	charcoal	4	central	1:7000
71	cation exchange	charcoal	5	not N terminus	1:125,000
72		second antibody	0.5-2.5	N terminus	1:20,000
73	cation exchange	second antibody	10		1:5000
74	gel filtration	charcoal	7.8-15.6		1:84,000
75	gel filtration	charcoal	10		1:5000
76	gel filtration	second antibody charcoal	1.6	central (ref 72)	1:7000-1:10,000
77	cation exchange	second antibody	25 pg/ml		1:80,000
78	cation exchange	charcoal	0.3		1:12,500
79	gel filtration	charcoal	10 pg/ml		1:8000
80	hydrophobic chromatography	second antibody	10 pg/ml	central	1:150,000
81	cation exchange	charcoal	0.1-0.5		1:10,000
82	gel filtration	charcoal	1.25	N terminus	1:12,000
83	cation exchange	charcoal	3.9		1:2500
84	cation exchange	charcoal	2.5	central	1:10,000

(Table 2,87). Both the lactoperoxidase and chloramine T methods of iodination have proven successful. The availability of synthetic analogs has made possible determination of the regions of the somatostatin molecule with which antisera react. In cases where this has been studied, most antisera have recognized the central portion of the molecule although two antisera have bound the N terminus. Immunological cross-reactivity of somatostatin with other known peptides has not been described in spite of the fact that somatostatin shares structural homology of amino acid residues 10 to 13 with secretin and glucagon (residues 5 to 8), gastric inhibitory polypeptides (residues 5,6 and 8) and vasoactive intestinal polypeptide (residues 6 and 7).

Nakagawa and colleagues (88) have reported an enzyme-linked immunoassay that used somatostatin conjugated to alkaline phosphatase as the labelled antigen. The minimum detectable concentration of this assay was 40 pg per tube, more than that for the RIAs.

2. Bioassay

Vale *et al* (89) developed a bioassay for somatostatin that exploits its GH inhibiting effect on rat pituitary cells in culture. This bioassay has been validated by being used for the original isolation of somatostatin. Essentially, a standard dose-response curve comparing the amount of GH released in the presence of varying concentrations of synthetic somatostatin is constructed and the concentration of somatostatin-like biological activity in an experimental sample is derived from the standard curve after having measured the amount of GH released in the presence of the sample. Further consideration will be given to this bioassay in the experimental section.

C. ANATOMICAL LOCALIZATION

1. Nervous System

a. Radioimmunoassay studies. Several studies have documented the widespread anatomical distributions of immunoreactive somatostatin (IRS) in the brain of the rat (Table 3). Vale *et al* (Table 3, 92) have shown a comparable distribution in the rat brain of somatostatin-like biological activity as determined by a cultured pituitary cell bioassay. The highest concentrations of IRS are found in the ME and medial basal hypothalamus

Table 3. Anatomical Distribution of Radioimmunoassayable and Bioassayable Somatostatin in the Rat Central Nervous System

	Bioassay Somatostatin ng/mg wet weight	Radioimmunoassay						
		IRS ng/mg wet weight				IRS ng/mg protein		
Reference								
Region	92	90	76	71	73	90	76	91
Median Eminence					15.5	309	248	
Hypothalamus	4.7	2.12	2.3	1.105	1.397		26	73
Preoptic area						8.4		4.3
Septum		} 0.64		} 0.522		2.1		1.9
Thalamus	0.5	0.15		0.464				0.5
Striatum	0.8	0.05		0.281				
Amygdala	2.1				0.35-0.27	3.9		3.7
Hippocampus	0.6					1.1		0.5
Cerebral cortex	0.9-2.4	0.03	0.52	0.098	0.093	2.4	6.7	0.7-1.6
Olfactory bulb		0.02	0.08	0.152		6.3	1.07	0.5
Cerebellum	0.2	0.02	0.03	0.047			0.43	0.4
Brainstem	2.5	0.05	0.44	0.135		0.9-3.3	5.1	0.9-2.4
Spinal cord	5.0		0.67	0.453	0.114		10.4	2.4
Neurophypophysis							5.6	
Pineal						0.5	0.14	0.3

with substantial levels also present in the preoptic area, amygdala (93), and spinal cord. IRS concentration is low in the cerebellum and pineal gland. Brownstein *et al* (90) determined that the ME, arcuate nucleus, periventricular nucleus, ventral premammilary nucleus, and ventromedial nucleus contained the highest IRS concentrations of several hypothalamic nuclei tested. The circumventricular organs of the rat also contain IRS (94) in addition to the other hypothalamic releasing hormones, TRH and LHRH (95).

The regional distribution of IRS in the human brain bears basic resemblance to that in the rat with highest levels found in the hypothalamus, intermediate levels in the cerebral cortex, brainstem, and spinal cord, and low concentrations in the cerebellum (96). Fetal human brain obtained from 10 to 22 weeks of gestation contained IRS with concentrations in the cerebral cortex being 25 percent of those in the hypothalamus (97). An increase of hypothalamic IRS concentration occurred with gestational age. By immunohistochemical methods IRS was detected in the human hypothalamus as early as 16 weeks of gestation (99).

IRS as determined by RIA and immunohistochemistry has been detected in the central nervous system of all vertebrate classes tested, including Cyclostomata (Table 4). In addition, a RIA study has shown the presence of IRS in a peripheral nerve, the vagus of dogs and cats (157). In the rat hypothalamus IRS was detectable at 2 days of life, increased to a peak concentration at 28 days, and declined thereafter (158). An inverse relationship existed between the hypothalamic IRS and serum GH concentrations during this period.

Subcellular fractionation studies of rat hypothalamus, preoptic area, amygdala, thalamus, striatum, and cerebral cortex indicated that 60-70% of IRS was localized in the synaptosomal fraction (73,159). Synaptosomes from the rat ME contained IRS by immunohistochemical staining in dense granules 90-110 mm in diameter (160). These observations suggested that IRS in the rat brain was predominantly localized in nerve terminals.

b. Immunohistochemical Studies.

i. Hypothalamus. Immunohistochemistry has proven particularly valuable in defining the localization and pathways of neuronal elements containing IRS. Investigators using various antisera and immunofluores-

Table 4. Phylogenetic and General Anatomical Distribution of IRS

H - hypothalamus
EB - extrahypothalamic brain
WB - whole brain
P - pancreas
P*- pancreatic islet organ
GI - gastrointestinal tract

	RIA		Immunohistochemistry	
Animals	Location	References	Location	References
VERTEBRATA				
MAMMALIA				
human	H,EB,P,GI	77,78,96-98	H,EB,P,GI	99-107
rat	See Tables 3 and 5		H,EB,P,GI	101,106-123
dog	H,EB,P,GI	28,78	H,EB,P,GI	107,124-128
mouse	H,P,GI	129-131	H,EB	124,132
pig	H	5,133	H,P,GI	101,103,107,108
sheep	H	72,134	H,P	101,108
ox			H,P	101,108
hamster	H,P,GI	86		
guinea pig			H,EB,P	123,132,135,136
tupaia	H,EB,P,GI	28,87		
monkey	P,GI	28	EB	132
rabbit			P,GI	107
fox			H,EB	137
cat			EB,P,GI	107,132
AVES				
chicken			H,P,GI	101,107,108, 138,139
pigeon *(unspecified)*	H,EB,P,GI	72	P	141
(Columbia livia)	H,EB,P,GI	140		
duck			H	142
quail *(Coturnix c. Japonica)*			GI	122

Table 4. Phylogenetic and General Anatomical Distribution of IRS (Con't)

Animals	RIA Location	RIA References	Immunohistochemistry Location	Immunohistochemistry References
AMPHIBIA				
frog *(unspecified)*	WB,P,GI	72		
(Rana esculenta)	H,EB,P,GI	143		
(Rana temporaria)	P,GI	144		
toad *(xenopus laevis)*	H,EB,P,GI	140,145		
tadpole *(Alytes obstetricans)*			H	146
newt *(unspecified)*			H	108
REPTILIA				
lizard *(Lacerta muralis)*			H	147
(Anolis carolinensis)			P,GI	122,148
tortoise *(Chersine angulata)*	H,EB,P,GI	140		
snake *(Vipera berus)*	P,GI	144		
(Natrix Natrix)	P	144	P	144
OSTEICHTHYES				
trout *(Salmo gairdeneri)*			H,P,GI	108,149,150
catfish *(unspecified)*	WB,P,GI	72		
(Ictalurus punctata)			P*	151
daddy sculpin *(Cottus scorpius)*	P*	144	P*	144
chichlid *(Sarotherodon mossambicus)*	H,EB,GI	140		
mudsucker *(Gillichthys mirabilis)*			P	122
anglerfish *(Lophius americanus)*	P*	152	P*	151
CHONDRICHTHYES				
torpedo *(unspecified)*	WB,P,GI	72		
ratfish *(Hydrolagus colliei)*	WB	153		
dogfish *(Squalus acanthias)*	WB,P,GI	144,153		
(Poroderma africanum)	H,EB,P,GI	140		

Table 4. Phylogenetic and General Anatomical Distribution of IRS (Con't)

Animals	RIA		Immunohistochemistry	
	Location	References	Location	References
CYCLOSTOMATA				
hagfish *(unspecified)*	WB,P	72		
Atlantic hagfish *(Myxine glutinosa)*	P*,GI	144	P*	154
Pacific hagfish *(Eptatretus stouti)*	P*,GI	155	P	122
lamprey *(Lampreta fluviatilis)*			P	154
INVERTEBRATA				
TUNICATA				
sea squirt *(Ciona intestinalis)*	GI	144	GI	156

cence or immunoperoxidase methods have observed IRS in nerve terminals of the ME of the rat (106,108-116) guinea pig (135,136), dog (124,125), mouse (124), fox (137), duck (142), pig, sheep, chicken, trout (108), lizard (147), amphibians (108,146), and human (99,100) by light microscopy. The external zone of the ME contained the highest concentration of fibers staining for IRS which were often in close proximity to capillaries of the pituitary portal plexus. Electron microscopic studies (115,161) have confirmed these observations and have identified the IRS in secretory granules 90-110 nm in diameter. Immunohistochemical staining for IRS and LHRH in the same microscopic sections of rat ME revealed that these peptides existed in two anatomically distinct neuronal systems with no fibers containing both peptides (162). Cell bodies that stain for IRS are found predominantly in the preoptic and anterior periventricular hypothalamus (99,106,111,115-118,125). Secretory granules that stain for IRS and are of a similar size to those in nerve terminals (161) and synaptosomes (160) of the ME are present in neurons of the periventricular nucleus by electron microscopy (163). Perikarya, including in some cases the magnocellular neurons, that stain for IRS have been identified in the paraventricular and/or the supraoptic nuclei of the rat (164,165), human (99), fox (137), duck (142), and lizard (147). These studies, however, contain conflicting evidence whether somatostatin and vasopressin are localized in the same neurons. De Vitry *et al* (166) have reported only slight staining for IRS in a cloned mouse neuronal cell line that synthesizes vasopressin and neurophysin, but intense staining for IRS in a different mouse hypothalamic line. Hoffman and Hayes (125) demonstrated somatostatin immunohistochemically in small neurons of the dog paraventricular nucleus, but not in the magnocellular neurons. IRS-positive fibers are also present in the neurohypophysis (99,111,125,142,146,147,149). Staining for IRS occurs in occasional neuronal perikarya in the arcuate nucleus (106,115,125) and in fibers in the arcuate, ventromedial and ventral premammillary nuclei (111,125). IRS has not been identified in tanycytes (106,113,142).

ii. Extrahypothalamic nervous system. Cellular elements which contain IRS are identifiable in the circumventricular organs, other extrahypothalamic brain sites, spinal cord, primary sensory neurons, sympathetic ganglia, and nerves intrinsic to the gut. The circumventricular organs, which afford a site of molecular exchange through fenestrated capillaries between the brain and the vascular system and whose functions are poorly understood (167), contain IRS in two distinct cellular elements. Neuronal fibers and endings which stain for IRS are found predominantly close to capillaries in the organum vasculosum of the lamina terminalis (OVLT) (106, 109,111,113,114,116,168,169), subfornical organ (111,116), and pineal gland (109,114). Electron microscopy revealed IRS-positive secretory granules 90 to 120 mm in diameter in nerve terminals of the OVLT (114,168). Ependymal and subependymal cells contained IRS in the subcommissural organ (109,114) area postrema and subfornical organ (114). Neuronal cell bodies which stain for IRS are present in the zona incerta, bed nucleus of the stria terminalis, cortical amygdaloid nucleus, hippocampus, pyriform, entorhinal, and neocortex (170,171) and the reticular nucleus of the medulla oblongata (132). Nerve fibers or terminals that are positive for IRS are located in the interstitial nucleus of the stria terminalis, nucleus accumbens, medial portion of the caudate nucleus, amygdala, olfactory tubercle, several cortical areas, and the parabrachial nucleus of the brainstem (170,171). Others (172) have identified IRS-positive structures with the appearance of synaptic terminals that surround particular neurons throughout the neocortex, envelop CA1 and CA2 pyramidal neurons of the hippocampus, and diffusely surround neurons of the reticular nucleus of the thalamus. Proteolytic treatment of sections of tissue with pronase resulted in a widespread enhancement of staining for IRS in neurons in several rat brain regions (173). Johansson (174) has localized IRS to the Golgi apparatus of neurons in the central and peripheral nervous system.

A system of primary sensory neurons which stain for IRS exists in the rat. Dorsal root ganglia contain small cells positive for IRS which are distinct from cells containing substance P (175). Also IRS-containing fibers are visible in the dorsal horn of the spinal cord with the highest concentration being in Lamina II. Positive fibers occur in Lissauer's tract and

the adjacent part of the lateral funiculus. Forssmann (176) has identified IRS-positive fibers distributed extensively in the grey matter of the mammalian spinal cord including the vicinity around the central canal (lamina X), the intermedio-medial nucleus and the lateral column (laminae VI and VII). Neurons in peripheral sympathetic ganglia of the guinea pig and rat stain for both IRS and dopamine beta hydroxylase suggesting they are noradrenergic in nature (177). This observation supports the hypothesis that a neuron can contain more than one neurotransmitter, assuming that such a role exists for somatostatin.

Nerve fibers staining for IRS are present in the lamina propria and muscular layers of the mucosa, the myenteric plexus, and the submucosa of the rat small and large intestine, but not the stomach (111,178). Neuronal cell bodies positive for IRS are present in the myenteric and submucous plexes of the guinea pig ileum (179). Denervation of the ileum reduced the IRS content of the external muscle layer by one third but did not change the mucosal-submucosal content (179). Tissue cultures of small intestine from fetal mice studied after three weeks of growth contained fibers positive for IRS (180). These observations provide evidence for the existence of somatostatin-containing neurons which are intrinsic to the intestine.

c. Neuronal projections containing IRS. The existence of a neuronal pathway from cell bodies in the anterior hypothalamic periventricular area to the external zone of the ME has been postulated based upon direct visualization by immunohistochemical methods (116,170,171) and lesion studies. Placement of lesions in the periventricular nuclei or transection of nerves connecting the anterior hypothalamic area and the ME resulted in loss of nearly all IRS staining in the ME (170,171). In addition electrolytic lesions of the anterior hypothalamic-preoptic area (181-183) and complete (181,184) or anterior (182,184) deafferentation of the medial basal hypothalamus all reduced IRS concentrations in the ME by over 75% as determined by RIA. The IRS concentration in extra-hypothalamic brain regions was not affected in these experiments (181,184). Likewise, lesions in the amygdala did not change IRS concentrations in the medial basal hypothalamus (181) which indicates independence of the content of hypothalamic and extrahypothalamic somatostatin. A projection from the supraoptic and paraventricu-

lar nuclei to the neurohypophysis has been proposed on the basis of studies on several species (99,137,142,147,164). In support of this pathway, electrolytic lesions of the anterior hypothalamic area reduced the neurohypophyseal concentration of IRS by 82 percent (183).

Krisch (116) has described in detail by immunohistochemical methods the projection of IRS-containing fibers from the hypothalamic periventricular perikarya to: 1. hypothalamic nuclei including the preoptic, suprachiasmatic, ventromedial, arcuate, and premammilary nuclei, 2. rostral structures such as the OVLT, subfornical organ, olfactory tubercle, and the interstitial nuclei of the stria terminalis, 3. the amygdala *via* the stria terminalis and descending ventrolateral projections, and 4. the ME by descending projections.

d. Hormonal effects on IRS concentration in the brain. Several studies indicate that the concentration of IRS in the hypothalamus and some other brain regions is sensitive to hormonal manipulations. Hypophysectomized rats maintained for 28 to 133 days show a substantial depletion of IRS in the ME as determined by immunohistochemistry (185). In addition, RIA studies have indicated a reduction of IRS levels in the stalk-median eminence (SME) (186,187), hypothalamus (188,189), and septal-preoptic area (190) of rats sacrificed 12 to 28 days after hypophysectomy. In contrast, other studies (190,191) have observed no statistically significant change in the IRS levels of the rat hypothalamus and several extrahypothalamic brain regions two weeks after hypophysectomy. Administration of GH to rats following hypophysectomy has inhibited the depletion of IRS in the SME and hypothalamus as determined by RIA (187,188) and in the ME as determined by immunohistochemistry (192). Treatment of normal rats with GH increased the SME and hypothalamic concentration of IRS measured by RIA (187,188). These observations provide a possible mechanism for feedback control of GH on itself that is mediated by the GH effects exerted on tissue somatostatin. It is not known at present whether the effects of GH on hypothalamic IRS are mediated directly by GH (short loop feedback) or indirectly by the somatomedins (long loop feedback) (187).

Other hormonal effects on brain IRS are of uncertain significance. Acute administration of insulin to urethane-anesthetized rats caused an

increase of the hypothalamic, thalamic, and cortical IRS concentration within 15 minutes (193). No significant changes of hypothalamic IRS occurred after glucose or glucagon injection. Hypothalamic content of IRS showed no significant change in rats subjected to: i. thyroidectomy (189, 190), ii. treatment with thyroxine, or iii. thyroidectomy plus treatment with thyroxine (189). An increase in concentration of IRS in the rat striatum occurred after thyroidectomy (190).

2. Gastrointestinal Tract/Pancreas

Following upon the observation by Koerker *et al* (194) that exogenously administered somatostatin suppressed the plasma concentrations of insulin, glucagon, and glucose, Arimura *et al* (195) first demonstrated IRS by RIA in the rat pancreas and gastrointestinal tract. A large number of groups have confirmed the presence of IRS in these tissues of many animal species. Table 5 shows the distribution of immunoassayable IRS in the rat and human gastrointestinal/pancreatic tissues. Generally, IRS is present throughout the length of the gut with the highest levels being in the gastric antrum and the pancreas. The isolated rat pancreatic islets contain the highest IRS concentration of any tissue in this species. Results of similar anatomical mapping studies of IRS done on dog (28,78), monkey, and tupaia (28) tissues are consistent with the distribution shown in table 5. Table 4 indicates the phylogenetic and general anatomical distribution of IRS determined by RIA and immunohistochemistry in the gastrointestinal/pancreatic tissues of a wide variety of animals. Among the lower species IRS is generally less abundant in the gut than in the pancreas (122,144). One invertebrate, *Ciona intestinalis*, or the sea squirt, contains IRS by RIA and immunohistochemistry in its gastrointestinal tract (144,156) although other invertebrates studied, including marine arthropods, molluscs, starfish, and amphioxus, do not contain IRS (144). Somatostatin is therefore likely a phylogenetically ancient peptide although the origin of insulin probably predates that of somatostatin considering that insulin occurs in some invertebrates that do not contain somatostatin (144).

There is general agreement based on light and electron microscopic immunohistochemical studies that IRS is localized to the D cells of the

Table 5. Distribution of IRS in Gastrointestinal Tissues

	IRS ng/mg wet weight					IRS n g/mg Protein		
References	195[a]	76	78	77	98	195	71	76
Region								
RAT								
pancreas (whole)	0.143	0.17	0.033			33.8	26.5	1.8
pancreas (islets)								786
stomach (whole)		0.37						2.4
stomach (body)			0.046				1.66	
stomach (fundus)	0.463					9.5		
stomach (antrum)	0.426		0.050			9.7	20.2	
duodenum (whole)		0.15	0.017				2.17	1.94
duodenum (upper)	0.033					1.6		
duodenum (lower)	0.021					1.3		
jejunum	0.03	0.07				1.6	2.39	1.94
ileum							5.22	
colon							15.36	
HUMAN								
pancreas			0.253		0.558			
stomach (body)			0.294		0.146			
stomach (fundus)				0.60				
stomach (antrum)			0.465	1.68	0.508			
duodenum (whole)					0.345			
duodenum (upper)				1.35				
jejunum					0.181			
ileum					0.066			
colon					0.033			

[a] calculated from data given in reference 195.

pancreatic islet and gastrointestinal mucosa (103-105,107,141). In the rat IRS is concentrated in D cell secretory granules that are of low to moderate electron density, have a limiting membrane closely applied to the granule core (120), and have ranged in diameter from 150-210 nm (119,121, 107). The diameter of IRS-positive secretory granules was similar in pancreatic and gastric tissue obtained from the same species but differed greatly from one species to another (107). D cells from the pancreas and stomach of humans, chickens and cats contain distinct IRS-positive secretory granules that are larger in diameter than those of rat D cells (105, 107).

Species differences exist in the distribution pattern of D cells in the pancreas and gastrointestinal tract (107). For instance, in rabbits and pigs the oxyntic gland area of the stomach contains a higher concentration of D cells than the pyloric region, whereas the reverse applies in the dog, cat and human (107). In the pancreatic islet of the rat, chinese hamster, mouse, and human the D cells are localized predominantly at the periphery of the islet as a sheath just interior to the A cell layer and exterior to the central B cells (196). In the horse islet the A and D cells are more centrally localized. This architectural arrangement causes the somatostatin-containing D cells to occur in close proximity to a higher fraction of the A cells than B cells, a fact which has relevance to a potential paracrine intra-islet function for somatostatin. The D cells of the gastric glands of rats and humans have elongated cytoplasmic processes which traverse the gastric glands and terminate on gastrin-containing and hydrochloric acid-producing cells (197). These processes may provide a pathway for the local release of somatostatin onto particular cells of the gastrointestinal glands.

Studies of fetal tissue have demonstrated the presence of IRS at early stages of development, for instance 14-17 days of gestation in the rat pancreas (107,198) and 16-17 days of gestation in the rat duodenum (107). By birth the rat pancreas contained a concentration of IRS which approximated that in the adult pancreas. Explanted rat embryonic pancreases maintained in organ culture independent of nervous input preserved their content of IRS which suggests that innervation is not a crucial factor in

determining pancreatic IRS content (198). The embryonic chicken pancreas contained IRS by immunohistochemistry by 3.25 days of gestation when the pancreatic bud first appeared (138). Cells positive for IRS by immunohistochemistry have been observed in the pancreas, stomach and intestine of human fetuses as early as 15 weeks of gestation (107).

3. Other Tissues

Studies using RIA have detected IRS in thyroid extracts from rats (71,199) and rabbits (200) and have shown that thyroid IRS elutes in a similar volume as synthetic somatostatin on gel filtration chromatography (200). Immunohistochemical investigations have revealed IRS in the parafollicular C cells of the rat (106,111), rabbit (200), dog and human (202) thyroids although other studies have failed to demonstrate IRS immunohistochemically in the dog (200) and normal human (200,204) thyroids. Thyroid parafollicular cells that contain both IRS and immunoreactive calcitonin have been observed in the rat (201,203) and rabbit (200). Two groups have demonstrated IRS in human thyroid medullary carcinomas (204, 205).

Lundberg *et al* (206) have detected a low number of cells staining for IRS by immunohistochemistry in the normal human adrenal medulla and a low to moderate number in two out of three human pheochromocytomas. One study (71) observed a low concentration of IRS by RIA of rat adrenal gland extracts although two other groups (76,195) failed to confirm this finding.

The chicken thymus contains radioimmunoassayable IRS at a similar concentration to that found in the chicken intestine and contains cells that show immunofluorescence due to IRS (207). The thymic IRS is detectable two days after hatching but not before. It elutes similarly to synthetic somatostatin on gel filtration and ion exchange chromatography. Studies of rat thymus have noted low or undetectable levels of IRS by RIA (71,195).

Kumasaka *et al* (83) detected IRS by RIA and immunofluorescence methods in the chorionic villi and decidua of human placentas studied at 5-12 weeks of gestation. The cytotrophoblast contained a higher intensity

of IRS immunofluorescence than the syncytiotrophoblast. In contrast, Fitz-Patrick and Patel (208) detected no IRS by RIA of human placentas at 14, 18 and 36-40 weeks of gestation.

4. Body Fluids

a. Blood. Several studies have detected IRS in the blood of rats, dogs and humans although controversy exists about the optimum RIA procedure and the true concentration of IRS. As is evident in Table 6, investigators have used a wide variety of procedures to process blood samples, ranging from no extraction (209-211,213) to a complex method of acetic acid, acetone and organic solvent extraction (215). Major discrepancies are evident in Table 6 with respect to the concentration of IRS in human blood and rat peripheral and hepatic portal blood. Nevertheless, IRS levels are consistently higher in hepatic and hypophysial portal blood than in central blood pools, an observation which is consistent with the fact that these portal vessels drain organs enriched in IRS content. It is not possible at this time to completely resolve the discrepancies of IRS concentration observed by different groups. However, an attempt can be made to indicate the potential problems faced when one tries to measure IRS in blood. These factors may account for the variety of results reported in the literature:

i. Somatostatin binding proteins. Conlon *et al* (216) have demonstrated by gel filtration chromatography at neutral pH the presence of a 150,000-200,000 molecular weight binding protein in dog plasma. When the authors conducted gel filtration at pH 2.5 no evidence for the binding protein was found, suggesting that a noncovalent association between IRS and the binding protein had been disrupted. Interestingly, an antiserum directed against the N terminus of somatostatin did not react with the IRS-binding protein complex which suggested that somatostatin associated with the binding protein through its N terminus region. Evidently, an N terminus-directed antiserum would measure a lower IRS concentration in plasma than a centrally-directed antiserum which would recognize both free and protein-bound IRS. In another study (70), rat plasma subjected to gel filtration at neutral pH revealed one predominant peak of IRS

Table 6. Radioimmunoassay Studies of Blood Somatostatin

Reference	Authors	Blood collected in	Extraction Method in	Species
209 210	Harris et al Schusdziarra et al	EDTA, aprotinin	none	dog
211	Kronheim et al	aprotinin	none	human
80	Penman et al	aprotinin	silica glass	human
212	Patel		acid ethanol	rat
70	Arimura et al		acetone	rat
213	Berelowitz et al	aprotinin		rat
214	Abe et al		acetone/acetic acid	rat
84	Gillioz et al		ethanol	rat
215	Chihara et al	acetic acid	acetone	rat

Table 6. Radioimmunoassay Studies of Blood Somatostatin (Con't)

Reference	Anesthesia	Sample	Region	Normal Concentration IRS pg/ml
209,210	none	plasma	peripheral vein	120
			hepatic portal vein	275
211	none	serum	peripheral vein	274
80	none	plasma	peripheral vein	17-81
212	ether	plasma	hepatic portal vein	310
			inferior vena cava	43
70	chloral hydrate	plasma	hepatic portal vein	29.6
			peripheral vein	10.5
213	ether	serum	aorta	304
			hepatic portal vein	523
			hepatic vein	290
214	urethane	plasma	hypophysial portal vessels	778
84	pentobarbital	plasma	hypophysial portal vessels	158
215	urethane	whole blood	hypophysial portal vessels	503
	urethane	whole blood	jugular vein	19.1
	pentobarbital	whole blood	hypophysial portal vessels	113
	pentobarbital	whole blood	jugular vein	18.1
	Althesin	whole blood	hypophysial portal vessels	155
	Althesin	whole blood	jugular vein	15.5

eluting at an earlier volume than synthetic somatostatin eluted. The early-eluting peak was interpreted to be a binding protein-IRS complex because of the observation that exogenous somatostatin added to plasma resulted in a similar elution profile to that of endogenous plasma IRS. Kronheim *et al* (217) have corroborated the existence of a binding protein in blood by ultracentrifugation sedimentation studies of somatostatin incubated with human serum.

In an early study Arimura *et al* (68) observed that charcoal removed 99% of [^{125}I-Tyr^{1}]somatostatin added to rat plasma but that charcoal treatment did not prevent cross-reaction of the plasma in the RIA. This is consistent with the demonstration by Ogawa *et al* (218) that rat and human plasma showed very low (1.2%) specific binding of [^{125}I-Tyr^{1}]somatostatin using charcoal to separate bound and free label. If the observations on dog plasma binding protein apply to the rat and human, then [^{125}I-Tyr^{1}]somatostatin would not be expected to associate with the binding protein (and therefore not to charcoal) because [^{125}I-Tyr^{1}]somatostatin is considerably modified at the N terminus compared to somatostatin. However, alternative explanations for the observations of Arimura *et al* (68) using charcoal could be: 1. nonspecific interference by charcoal-washed plasma in the RIA, such as by degrading the [^{125}I-Tyr^{1}]somatostatin resulting in an apparent inhibition of label binding in the RIA. 2. the existence of a large molecular weight covalently-linked form of IRS, such as a biosynthetic precursor, that does not bind to charcoal.

ii. Degradation by blood of somatostatin and [^{125}I-Try^{1}]somatostatin. Most studies acknowledge this effect but differ as to the conditions required to inhibit the degradation. Two groups (209-211,213) claim aprotinin (a peptidase inhibitor) alone or with EDTA (ethylenediamine tetraacetic acid) is satisfactory but others require extraction of plasma to prevent degradation effects (70,212,215). The half life of exogenously administered somatostatin is relatively short, in the range of 2 min (see below).

iii. Large molecular weight species of IRS. Patel (212) observed a large molecular weight form of IRS from rat hepatic portal and inferior vena caval plasma that eluted at the void volume of Sephadex G-25 columns

run in 6 mol/l urea to dissociate noncovalent bonds. Kronheim *et al* (211) also found a void volume peak of IRS on Sephadex G-25 acid chromatography of peripheral venous serum from a small subgroup of humans studied. Incubation of this void volume material in 6 mol/l urea for 24 hours followed by rechromatography did not change its elution position. However, chromatography of serum from the same subgroup of subjects on Sephadex G-50 to achieve greater resolution at larger molecular sizes revealed two peaks of IRS that eluted before synthetic somatostatin, one at the void volume and the other at an intermediate volume. Serum from the majority of human subjects revealed only a single peak on Sephadex G-25 chromatography. Similarly, Berelowitz *et al* (213) observed that the majority of IRS in rat plasma from various anatomical sites eluted as a single peak similar in position to that of synthetic somatostatin. Gel filtration under acid conditions of rat hypophysial portal plasma (84) showed a small peak of IRS that eluted just before synthetic somatostatin although the significance of this peak is suspect because a similar peak could be produced by addition of exogenous somatostatin to dog plasma followed by gel filtration chromatography in acid (216). Chihara *et al* (215) noted four peaks of IRS on ion exchange chromatography of a rat hypophysial portal blood extract. It is unlikely that the peaks reflect binding proteins because synthetic somatostatin added to peripheral plasma eluted as a single peak coincidentally with synthetic somatostatin. The precise nature of the three species of IRS with altered charge is unclear though.

A possible explanation for the variety of results obtained with respect to heterogenous forms of IRS in blood is that the antisera used may have differed in their ability to recognize larger forms of IRS. For instance, Vale *et al* (72) have shown that an N-terminus-directed antiserum reacted less well with a large hypothalamic form of IRS than did a centrally-directed antiserum. In addition, the various extraction procedures used may have differed in their ability to solubilize and recover heterogenous forms of blood IRS.

b. Other body fluids. Radioimmunoassayable somatostatin is detectable in cerebrospinal fluid (CSF), urine, amniotic fluid and pancreatic secretions. CSF from normal humans was shown to contain means of 35.4 and 55 pg IRS/ml in two studies (96,219). Patel *et al* (96) found increased levels of IRS in a variety of neurological disorders with very high concentrations being present in two patients with brain tumours. The possibility that the IRS concentration in CSF may be an indicator of nervous system disease merits further investigation. Human urine contained IRS at a concentration of 66 pg/mg creatinine (220). It migrated similarly to synthetic somatostatin in several chromatographic systems. Fitzpatrick and Patel (208) demonstrated IRS in human amniotic fluid with mean levels at 15-20 weeks of gestation being 320 pg/ml and at 32-40 weeks being 70 pg/ml. The IRS eluted on gel filtration chromatography done under denaturing conditions with an apparent molecular weight of $\geq$5000 daltons. The authors argued for the fetus as being the source of amniotic fluid IRS because of the absence of IRS in the placenta in their study, the absence of an arteriovenous gradient of IRS in cord blood and the presence of different concentrations of IRS in the amniotic fluids of twins with a monochorionic diamniotic placenta. IRS has been detected in dog pancreatic juice following stimulation of pancreatic secretion by intravenous infusion of secretin and the C terminal octapeptide of cholecystokinin (221). Gel filtration chromatography of the IRS revealed a major peak coinciding in elution volume with synthetic somatostatin and a minor early-eluting peak. Isoelectric focussing also showed two components of IRS with the major species appearing in the same pH range as synthetic somatostatin and the minor component focussing at a slightly higher pH.

D. BIOCHEMISTRY

1. Heterogeneity of Somatostatin-like Materials

Somatostatin isolated from sheep and pig hypothalamus (4,5) or pigeon and anglerfish pancreas (6,7) consisted of the identical tetradecapeptide amino acid sequence. In addition, Pradayrol *et al* (222) have isolated from pig intestine a 21 amino acid species with a heptapeptide extension on the N terminus of somatostatin.

a. Central nervous system. Studies using chromatographic methods suggest the presence in the nervous system of immunologically and biologically active somatostatin-like materials which have physicochemical properties different from those of somatostatin. During the original isolation of somatostatin from sheep hypothalami a fraction with GH release inhibitory activity was observed which was distinct from somatostatin (57). Vale *et al* (72) described an extraction fraction from sheep hypothalami which yielded two peaks on gel filtration chromatography. One peak eluted earlier than somatostatin and the other co-eluted with synthetic somatostatin. The early-eluting form showed reduced reactivity with an antiserum directed against the N-terminus of somatostatin which suggests that it may represent somatostatin with an extension or modification at the N-terminus. Millar (134) has reported that an extract of sheep hypothalami resolved into four peaks on gel filtration chromatography. Two peaks which preceded somatostatin did not change their elution positions after incubation under denaturing conditions. Pig hypothalamic extracts contain at least one (133) and possibly two (223) immunologically and biologically active forms of somatostatin-like material which are physicochemically distinct from somatostatin. King and Millar (140) demonstrated broad elution profiles on ion exchange chromatography of IRS from rat, pigeon, tortoise, frog and teleost fish hypothalamus and suggested the possible existence of heterogeneity of IRS. Patel and Reichlin (76) observed a form of IRS from rat hypothalamus and extrahypothalamic brain which eluted as a higher molecular weight material on gel filtration done under denaturing conditions. Spiess *et al* (224) identified two large species of IRS from rat hypothalami with apparent molecular weights of 12,500 to 11,000 and 4,500 to 3,500 daltons. (The molecular weight of somatostatin is 1638 daltons). These large species maintained their apparent molecular weights under dissociative and reducing conditions. Zingg and Patel (225) found large forms of IRS of 25,000 and 4,000 daltons in rat ME and neurohypophysis. Reduction of disulfide bonds and use of denaturing conditions during gel filtration chromatography did not alter their elution positions. Both forms of IRS were released from ME

and neurohypophysis *in vitro* in the presence of high extracellular potassium concentration. Gentle treatment of the 25,000 dalton species of Zingg and Patel and the 12,500-11,000 dalton species of Spiess *et al* with trypsin resulted in their partial transformation to a species with a similar gel filtration behavior as synthetic somatostatin. Mouse hypothalamic extracts also contained two forms of IRS which eluted as larger molecular weight species than somatostatin (131).

b. Gastrointestinal tract/pancreas. Gel filtration studies have revealed heterogeneity of IRS from the pancreas and gastrointestinal tract. Arimura *et al* (195) and Patel and Reichlin (76) demonstrated an early-eluting species of IRS on Sephadex G 25 chromatography of a rat stomach extract. Arimura *et al* also observed that the major species of IRS from rat pancreas eluted at the void volume. When this peak was incubated with 8 mol/l urea to dissociate noncovalent bonds and rechromatographed the elution volume of most of the IRS had changed to become identical to that of synthetic somatostatin (226). However, a portion of the early eluting peak did not alter its position of elution. Conlon *et al* (227) partially purified dog pancreatic IRS by affinity chromatography and observed two large species of IRS of 12,000 and 3,500 daltons on Biogel P 10 chromatography. The 12,000 dalton species changed its elution position to that of synthetic somatostatin after reduction of disulfide bonds by dithiothreitol. This observation suggests that the 12,000 species may represent somatostatin bound via a disulfide linkage to a nonimmunoreactive polypeptide. Heterogeneity of IRS extracted from human stomach, pancreas, and duodenum was mainly evidenced by a small void volume peak on Sephadex G 25 chromatography (77,78).

In a study of both extrahypothalamic and stomach IRS from dogs Zyznar *et al* (228) reported IRS species of 12,000, 3000 and 1600 daltons on gel filtration chromatography. All three species could be isolated from stomach or brain by affinity chromatography. The 12,000 dalton form had an acidic isoelectric point on isoelectric focussing but the 3000 and 1600 dalton forms had basic isoelectric points similar to that of synthetic somatostatin. The two smaller forms of IRS did not change their elution positions on gel filtration chromatography after incubation in 6 mol/l guanidine HCl and

dithiothreitol. In contrast, the 12,000 dalton species was incompletely transformed into a 1600 dalton species after similar incubation but not after incubation under dissociating conditions alone. The 1600 dalton forms from both dog brain and stomach inhibited gastric acid secretion in rats. IRS extracted from human pancreatic tumours that secrete somatostatin (somatostatinomas) has demonstrated heterogeneity on gel filtration chromatography. In one case the tumour IRS was stated to elute as two peaks corresponding to apparent molecular weights of 12,000 and 3000 daltons and a third peak coinciding in position with synthetic somatostatin (229). Plasma from this patient contained species of IRS that eluted at similar positions. Another pancreatic somatostatinoma produced IRS that resolved into four peaks on Sephadex G-25 chromatography, one of which coincided in position with synthetic somatostatin and another of which eluted after the column void volume (230). Four pools of fractions from this chromatography all inhibited insulin and glucagon secretion from perfused porcine pancreas although it is unclear from the description given in the report whether the four pools each represented a separate peak of IRS.

2. Biosynthesis

Noe and colleagues (152,231) have demonstrated biosynthesis of somatostatin by anglerfish pancreatic islets. They used radioactively-labelled amino acids in a pulse-chase protocol and analysed the labelled products by gel filtration chromatography, polyacrylamide gel electrophoresis, and RIA for somatostatin. A large species of IRS with a molecular weight of 8000 to 15,000 daltons was initially labelled followed in time by a smaller species which behaved immunologically, chromatographically, and electrophoretically like somatostatin. The large species was resistant to strong denaturing and reducing conditions but it was cleaved to the small species of IRS by incubation with trypsin. These studies strongly support a hypothesis that somatostatin is synthesized via a larger molecular weight precursor.

3. Degradation

a. Central nervous system. Study of the degradation of somatostatin has been restricted to the effects of brain fractions or partially purified

enzymes on synthetic somatostatin. Griffiths *et al* (232-234) have observed inactivation of IRS by cytoplasmic and particulate (microsomes and mitochondria) fractions of rat hypothalamus, thalamus, cortex and cerebellum. The cytoplasmic fraction produced the greatest degree of inactivation with a pH optimum of 7.3. The inactivation was eliminated by heating the fractions to 100°C. Treatment of rats with propylthiouracil for 90 days resulted in a two-fold increase in the activity of cytoplasmic peptidases from hypothalamus but not brain (235). This effect of propylthiouracil was reversed by treatment of the rats with thyroxine. Marks and Stern (236) have demonstrated that a neutral proteinase (Cathepsin M) prepared from rat brain cleaved somatostatin at three probable interior sites: between Phe^6-Phe^7, Trp^8-Lys^9, and Thr^{10}-Phe^{11}. The analogs dihydrosomatostatin (reduced disulfide bond) and des-(Ala^1,Gly^2)-N^3-Ac-somatostatin were also cleaved by this enzyme. An aminopeptidase of comparatively low activity also was present in crude brain extracts. The analog [D-Trp^8]somatostatin was resistant to endopeptidase activity in rat brain homogenates (237). An acid proteinase (Cathepsin D) purified from calf brain cleaved dihydrosomatostatin at the probable sites Phe^6-Phe^7 and Trp^8-Lys^9 (238). The analog [D-Trp^8]somatostatin was not cleaved, evidence in support of these two proposed cleavage sites. An independent study by Akopyan *et al* (239) demonstrated that Cathepsin D purified from bovine hypothalamus had endopeptidase activity on somatostatin.

b. Blood. The half-life of synthetic somatostatin infused intravenously is relatively short, 1.82 minutes in the dog (240) and 2.3 minutes in humans (241). Removal of somatostatin from blood by tissues probably accounts in part for its short half life. A transhepatic venous gradient for IRS has been observed in the rat (213) and dog (210) and a trans-renal gradient between the aorta and renal vein exists in the rat (242). In addition, demonstration of IRS in urine (220) and a low metabolic clearance rate for somatostatin in patients with chronic renal failure (241) are consistent with a renal mechanism contributing to removal of somatostatin from blood.

Rat serum and plasma and human serum also inactivate synthetic somatostatin *in vitro* (243,244). The inactivation of somatostatin by plasma was related to temperature, dose of plasma and time of incubation with synthetic

somatostatin (244). Also, the inactivation of somatostatin was eliminated by heating the plasma and was reduced by benzamidine, a proteinase inhibitor. Benuck and Marks (243) have identified in human serum an aminopeptidase and an endopeptidase as indicated by cleavage of the analog des-(Ala^1,Gly^2)-N^3-Ac-somatostatin. The fact that the analog [D-Trp^8] somatostatin was resistant to internal cleavage was consistent with an endopeptidase activity in serum. Somatostatin injected intravenously into rats was cleaved by an aminopeptidase as shown by isolation of the cleaved product and analysis of its amino acid composition (245,246).

4. Structure-Activity Relationships

Vale *et al* (247) and Holladay *et al* (248) have commented upon the difficulty of interpretation of data on the biological activity of peptide analogs in terms of structure-activity relationships. Structural modification of a peptide may affect biological activity by alteration of specific essential groups or perturbation of the molecular conformation. In addition, an analog can alter several potential biological events, including: i. distribution in body tissues and fluids, ii. enzymatic degradation, iii. receptor binding, and iv. receptor-analog complex activation of intracellular events. Since relatively little is known at present about these biological mechanisms with respect to somatostatin, discussion of structure-function relationships of somatostatin analogs has necessarily been predominantly descriptive rather than mechanistic.

a. Conformation. Holladay and Puett (249), on the basis of physical chemical studies, proposed that somatostatin exists in aqueous solution as an ellipsoid monomer with a conformation characterized by a hairpin loop of the peptide chain with four to six residues forming an antiparallel β-pleated sheet. In their model one end of the molecule is hydrophobic, containing Trp^8 at its extremity. The other end consists of the Ala^1-Gly^2 side chain and the disulfide bond and is hydrophilic. Amino acid substitutions of Phe^7 and Trp^8 greatly modify the conformation, substitutions of Phe^{11} have lesser effects, substitutions of Phe^6 and Thr^{12} have very slight effects. Holladay *et al* (248) interpreted these results as support for a β sheet structure which extends from residues 6 to 13 and involves hydro-

gen bonds between NH and CO groups of residues Phe^{6} and Ser^{13}, and Trp^{8} and Phe^{11}. Veber *et al* (250) and Arison *et al* (251) suggested that somatostatin at its cellular receptor site exists in a different conformation, characterized by hydrophobic interactions between the Phe^{6} and Phe^{11} moieties.

b. Activity of somatostatin analogs on GH release. Somatostatin and dihydrosomatostatin were equipotent with respect to inhibition of GH release *in vitro* (252) and *in vivo* (253). The possibility of interconversion of these forms under experimental conditions could not be ruled out however. The noncyclic analogs [$Ala^{3,14}$]somatostatin (254,255) and [S-Met-$Cys^{3,14}$] somatostatin (255) possessed low biological activity but this could not be solely attributed to their inability to form disulfide bonds. Veber *et al* (256) and Garsky *et al* (257) have demonstrated that cyclic analogs which do not contain sulfur possess significant biological activity. This indicates that the sulfur moieties are not specifically required for biological activity. Analogs with the side chain Ala^{1}-Gly^{2} removed (255), including those N-acylated at the Cys^{3} residue (258-261), retained biological activity, which shows that the cyclic portion of somatostatin enclosed by the disulfide bond possesses the structural requirements for biological activity. Additions of amino acids to the N terminus or blockage of the N terminus by acylation did not affect biological activity (262). Amidation of the C terminus reduced the potency by two thirds (262). Deletion of single amino acids revealed a greater than 30 fold reduction of *in vitro* biological activity in the cases of Phe^{6}, Phe^{7}, Trp^{8}, Lys^{9} and Phe^{11} indicating the importance of these residues for biological activity (247,262). Systematic replacement of each residue in the cyclic portion of somatostatin with its respective D-amino acid revealed that [D-Trp^{8}]somatostatin (247,263) and [D-Cys^{14}]somatostatin (247,262) had eight and 2.7 times the potency of somatostatin, respectively, although activity was reduced in the cases of the other substitutions. Studies of the effect of halogenation of the indole group of D-Trp^{8} resulted in the analogs [D-5F -Trp^{8}]somatostatin and [D-5Br-Trp^{8}]somatostatin which were 25 and 30 times more active, respectively, than somatostatin on GH release *in vitro* (264). Substitution of Trp^{8} by other groups including the aromatic

amino acid D-Tyr (262,265,266) decreased activity indicating the absolute requirements for the indole ring at position 8. Although [D-Trp^{8}]somatostatin is resistant to degradation by brain (237) and serum (243) enzymes, Vale *et al* (247) have argued that its high potency may not be due solely to this property because the analog was highly active *in vitro* under assay conditions in which degradation of somatostatin is not evident. Vale *et al* (247) have reported several seven to ten amino acid analogs of somatostatin whose activities, taken together with the above results, suggest a model for biological activity which requires: i) hydrophobic amino acids at positions 6,7,8 and 11, ii) lysine at position 9, iii) a spacer amino acid (Thr in somatostatin) at position 10, and iv) a cystine disulfide bond or additional amino acids to maintain the conformation of the critical region. Veber *et al* (250,267) maintained that the role of Phe^{6} and Phe^{11} in somatostatin is to stabilize the peptide conformation by forming hydrophobic bonds with each other and that amino acids 7 to 10 are responsible for receptor binding. In support of their formulation they have synthesized analogs consisting of amino acids 7 to 10 of the somatostatin sequence that are maintained in a constrained conformation within a bicyclic structure and exhibit enhanced biological activity.

c. Analogs with dissociated activities. Although early observations revealed concordant activities of analogs on release of GH, insulin and glucagon (268) many subsequent studies have discovered analogs with dissociated activities on secretion of GH, insulin, glucagon and gastric acid (247,262,269-271). In particular, analogs have been described which have the following relative potencies for inhibition of the release of the hormones indicated: 1. GH and insulin greater than glucagon (269,272-274), 2. GH and glucagon greater than insulin (275-278), although one study disagrees with this conclusion with respect to the [D-Cys^{14}]-substituted analogs (279), 3. GH greater than insulin and glucagon (257,280-282), 4. Glucagon greater than GH and insulin (283). More extensive discussion of this clinically important subject is beyond the scope of this thesis.

E. BIOLOGICAL ACTIONS

Because the studies described in this thesis primarily concern somatostatin localized in the central nervous system, the presentation of the biological actions of somatostatin will focus upon its effect on the secretion of anterior pituitary hormones and its action on neuronal function. Detailed discussion of somatostatin's action on non-pituitary hormone secretion, metabolism and gastrointestinal physiology would be beyond the scope of this thesis. Several reviews of these subjects are available for the interested reader (284-296).

1. Effects on the Secretion of Anterior Pituitary Hormones.

a. Growth hormone (GH). The inhibitory action of somatostatin on the secretion of GH has been established in several experimental models in addition to the anterior pituitary cell bioassay which contributed to its original isolation. By use of a pituitary perifusion model, somatostatin has been shown to inhibit basal secretion of GH and release stimulated by TRH, theophylline, dibutyryl cyclic-adenosine monophosphate (db cAMP), and high extracellular potassium concentration (297-300). A rebound release of GH occurred within a few minutes of removing the somatostatin (298,299). The basal plasma GH concentrations of rats determined at single time points or as a pulsatile secretory profile were reduced by somatostatin administration (301,302). Also, somatostatin inhibited the stimulation in rats *in vivo* of GH secretion due to pentobarbital (301,303, 304), morphine (303,305), TRH (306), chlorpromazine (307,308), isoprenaline (308), prostaglandin E_2 (303,309), pig SME extract (309), and electrical stimulation of the hypothalamic ventro-medial nucleus and the basal-lateral amygdala (310). With reference to other species, somatostatin inhibited GH secretion under the following conditions: 1. Stimulated *in vivo* by L-dopa in the dog (311), 2. Basal secretion *in vitro* and TRH-stimulated *in vitro* and *in vivo* in chickens (312,313). 3. Stimulated *in vivo* by prostaglandin E_1 (314), arginine (315,316), 3,5-dimethyl pyrazole (an antilipolytic agent), sodium propionate, glucagon, and glucose (316) and *in vitro* by prostaglandin E_1 (316) in sheep, 4. Basal secretion *in vivo* in fetal sheep (317), 5. Stimulated by prostaglandin $F_2\alpha$ *in vivo* in the bull (318),

6. Basal secretion *in vitro* in a teleost fish (319). In contrast to the above, intracerebral ventricular (icv) injection of somatostatin to rats anesthetized with urethane produced an increase in plasma GH levels (320).

Studies conducted on normal humans have observed either no effect (321,322) or a slight reduction (323) of basal plasma GH levels after administration of somatostatin. However, somatostatin convincingly inhibited the increase of blood GH provoked by sleep (324,325), exercise (326), and administration of L-dopa (321), arginine (321,327-331), leucine (331), insulin (328,332,333), ACTH (334), and bacterial pyrogen (335). Acromegalic patients have consistently shown suppression of basal plasma GH levels in response to somatostatin (322,327-329,332,336-342), as well as inhibition of the glucose-stimulated paradoxical rise of plasma GH in subjects in whom this phenomenon occurred (336,338). Acromegalic patients occasionally respond also to TRH and LHRH with an increase in serum GH while normal subjects do not. In one study (343) the TRH and LHRH stimulations of GH secretion were not affected by somatostatin; another study (344), using a higher dose of somatostatin, demonstrated inhibition of the TRH-induced response. Dwarfs with elevated immunoreactive GH levels (Laron's dwarfs) treated with somatostatin demonstrated suppression of basal plasma GH and inhibition of arginine-stimulated GH release (345,346). Somatostatin inhibited basal release of GH but not thyroid stimulating hormone (TSH), prolactin (PRL), luteinizing horomone (LH) or follicle stimulating hormone (FSH) from cultured human fetal pituitaries obtained from 10 to 18 weeks of gestational age (347). Human pituitary adenomas also displayed inhibition by somatostatin of basal (4,348) and theophylline-stimulated (349) release of GH.

The use of somatostatin in the therapy of acromegaly has been limited by its short biological activity *in vivo* and concern about its inhibitory action on secretion of hormones other than GH. Recently described analogs with prolonged activity and selective action on GH release may prove clinically applicable in the future.

b. Thyroid stimulating hormone (TSH). In contrast to its effect on GH secretion, somatostatin did not inhibit the basal release of TSH from cultured rat pituitary cells when incubated for less than five hours (350,

351). However, when incubated for three days somatostatin reduced the amount of TSH released into the medium and the total intracellular and extracellular content of TSH (350). Somatostatin inhibited stimulation of TSH release from cultured rat pituitary cells (350,351) induced by TRH, theophylline and high extracellular potassium concentration. Cycloheximide, an inhibitor of protein synthesis, did not block the effect of somatostatin on TRH-stimulated TSH release *in vitro* (350). Basal and TRH-stimulated accumulation of TSH in cultured mouse pituitary tumour cells and incubation media were reduced by somatostatin exposure for 24-72 hrs (352). Somatostatin administration to rats did not reduce basal plasma TSH levels (351) but inhibited the TRH-stimulated rise in plasma TSH (350,351). Thyroid hormone produced an additive effect with somatostatin on the inhibition of TRH-stimulated TSH release *in vitro* (350) and *in vivo* (350,351) in the rat. Basal TSH levels were not affected by somatostatin administration to bulls (318), but the TRH-stimulated increase in serum TSH was blocked by somatostatin in newborn sheep (353).

Somatostatin has either not affected (321,354) or only slightly reduced (323) basal serum TSH levels in humans during the day, but clearly reduced basal TSH levels during the night when these are normally highest (355). Stimulation of TSH release by TRH was inhibited by somatostatin in normal humans (330,332,354,356,357) and patients with primary hypothyroidism (330, 354). Somatostatin administration to acromegalics has not reduced basal TSH levels except in one patient who had a basal serum TSH level above normal (337,338). Elevated basal serum TSH in untreated primary hypothyroidism was reduced by somatostatin (358). Somatostatin also decreased elevated serum TSH levels in a hyperthyroid patient believed to have a TSH-secreting pituitary tumor (359).

c. Prolactin (PRL). Study of the effect of somatostatin on PRL secretion has produced different results depending on the experimental model used. Somatostatin inconsistently inhibited basal release of PRL from cultured pituitary cells from normal male rats (350,351,360,361) but more constantly suppressed secretion from cells of castrated or estrogen-treated male rats (350). With use of anterior pituitary cells from female rats both basal and TRH-stimulated secretion of PRL were inhibited (351).

Somatostatin administration to male rats did not affect basal or TRH-stimulated plasma levels of PRL (351). Elevation of plasma PRL levels in chronically cannulated rats after administration of the dopamine-depleting drug α-methyl paratyrosine or after hypothalamic lesions was not affected by infusion of large doses of somatostatin (250μg) - (Martin, J.B., personal communication). Basal release of PRL was not reduced by administration of somatostatin to cultured chicken anterior pituitary cells (313) and *in vivo* to sheep (314), bulls (318) and monkeys (362). Also, somatostatin did not inhibit PRL secretion stimulated *in vivo* by TRH, perphenazine or serotonin in the monkey (362) and prostaglandin $F_2\alpha$ in bulls (318), but did block PRL release stimulated by TRH in cultured chicken anterior pituitary cells (313) and *in vivo* by prostaglandin E_1 in sheep (314).

Somatostatin administration to normal humans has not affected serum PRL levels in the basal state (321,363), with the exception of one study (323), or stimulated by TRH (332,354,356,363), arginine (321), or insulin hypoglycemia (333). Some acromegalic patients have demonstrated a reduction of serum PRL by somatostatin (337,363) but others have shown no response (332,338,342,363). In one study two of five patients with a PRL-secreting pituitary tumor responded to somatostatin with a reduction of serum PRL (363). Somatostatin inhibited the release of PRL by combined TRH-arginine administration in two out of three patients with primary hypothyroidism (330).

d. Luteinizing hormone (LH) and follicle stimulating hormone (FSH).

Somatostatin has had no effect on basal (318,350), prostaglandin $F_2\alpha$-stimulated (318) or LHRH-stimulated (350) secretion of LH by rat anterior pituitary cells *in vitro* and bulls *in vivo*. Basal and LHRH-stimulated levels of LH and FSH in humans have not been affected by somatostatin administration (321,332) with the exception of a postmenopausal woman in whom basal serum LH was reduced (364). In males somatostatin has blunted the slight rise in serum FSH resulting from TRH administration (332). No effect on basal LH and FSH in acromegalics has been observed (337,338).

e. Adrenocorticotropic hormone (ACTH). Somatostatin has had no effect on basal release of ACTH from cultured pituitary cells from normal or adrenalectomized rats (365). However, stimulation of ACTH release by an

extract of rat SME and arginine vasopressin was inhibited, but only by use of cells from adrenalectomized rats and within a limited dose range of somatostatin.

In normal humans plasma ACTH concentrations were stable during somatostatin infusion (323) and somatostatin did not affect either ACTH (332) or cortisol (333) release stimulated by insulin hypoglycemia. Serum corticosteroid levels remained normal in acromegalics during a 28 hour infusion of somatostatin (338). In contrast, somatostatin significantly reduced elevated plasma ACTH levels in 6 patients with Nelson's syndrome (366,367) and 1 patient with Cushing's disease studied 16 days after bilateral adrenalectomy (367).

In contrast to the complexity of somatostatin's actions on anterior pituitary hormone secretion, it has had no effect on the blood concentrations of the neurohypophysial peptides arginine vasopressin in dogs (368) or neurophysin in humans (369).

2. Evidence for a Physiological Role in the Secretion of Anterior Pituitary Hormones.

Administration of somatostatin antiserum (SS-AS) *in vivo* or active immunization with somatostatin with the intention to neutralize endogenous somatostatin has provided evidence that somatostatin functions as a physiologically relevant inhibitor of GH secretion. SS-AS given to freely-moving cannulated rats partially reversed the suppression of pulsatile GH release resulting from the stress of swimming (370) or prolonged food deprivation (371). Suppression of serum GH induced by electro-shock stress in conscious rats was also prevented by SS-AS (85). SS-AS has elevated basal GH levels in conscious dogs (372) and urethane-and pentobarbital-anesthetized rats (373) and has increased the amplitude of peaks of episodic GH secretion in freely-moving rats (374). SS-AS abolished the inhibition of GH release after icv injection of substance P but did not alter the stimulation of GH release by opiate peptides given by the same route (375).

Active immunization of baboons against somatostatin resulted in an increase in the baseline levels of serum GH sampled over 12 hours and a diminution of the secretory bursts (376). The authors suggested that somatostatin may function in the baboon as a regulator of episodic bursts

of GH secretion and that a GH releasing factor was required to maintain a tonic influence on GH secretion. In support of this model, hypothalamic deafferentation (377) and electrolytic lesions of the medial preoptic area (378), which disrupted the somatostatin-containing projection to the ME, resulted in more frequent bursts of GH secretion and an elevation of trough GH levels in freely-moving rats. Anterior hypothalamic deafferentation and medial preoptic area lesions in rats also compromised the reduction of plasma GH due to stress (182).

Somatostatin may have an inhibitory role in determining peripheral blood TSH levels. SS-AS administration has increased basal TSH levels in conscious rats (374) and rats anesthetized with ether, urethane, or pentobarbital (373,379,380). The increase of blood TSH concentration resulting from cold exposure (374) or TRH administration (379-381) was potentiated by AS-SS. The effect of AS-SS to enhance basal and TRH-stimulated blood TSH levels persisted in rats treated with thyroid hormones or rats subjected to thyroidectomy (380,381) indicating a degree of independence of somatostatin action from thyroid hormone levels. However, surgical ablation of the medial basal hypothalamus of rats, which eliminated the influence of endogenous somatostatin on the anterior pituitary, prevented the stimulatory action of SS-AS on both basal serum GH and TSH levels (373). Cultured rat anterior pituitary cells have shown an increased basal release of TSH in the presence of SS-AS (382). Interestingly, the inhibitory action of somatostatin on basal and norepinephrine-stimulated TRH release from cultured rat hypothalami (383) provides another site of action of somatostatin for inhibition of TSH release in addition to its direct pituitary effect.

Consistent with the predominant lack of effect of exogenous somatostatin on plasma PRL levels *in vivo*, administration of SS-AS to rats did not alter basal serum PRL concentrations (373,379).

3. Mechanisms of Action on GH Secretion

a. Cyclic nucleotides. Pertinent studies have primarily investigated the relationship between somatostatin action and cyclic nucleotides, calcium ion flux, and cellular binding of somatostatin. Somatostatin has in-

hibited the release of GH stimulated by ^{db} cAMP (300,384,385), theophylline (384) and 3-isobutyl-1-methylxanthine (386) (both phosphodiesterase inhibitors) *in vitro*, and by ^{db} cAMP in humans (387). These results suggested that somatostatin may act on cellular mechanisms distal to cyclic adenosine monophosphate (cAMP). Somatostatin inhibited pituitary concentrations of cAMP in a basal state (388-391) and stimulated by theophylline (389), TRH (391), prostaglandin E_1 (388,391), prostaglandin E_2 (389), chlorpromazine (390), and 3-isobutyl-1-methylxanthine (386), all agents which stimulate the secretion of GH. Interestingly, similar structure-activity requirements have been shown for reduction of GH secretion and inhibition of cellular accumulation of cAMP by relatively few somatostatin analogs tested on rat pituitary cells *in vitro* (392). For instance [D-Lys^9]somatostatin had very slight or no action on GH release or cAMP accumulation.

Studies of the effect of somatostatin on pituitary guanosine 3',5'-cyclic monophosphate (cGMP) levels have reported conflicting results. Kaneko *et al* (391) demonstrated somatostatin stimulation of cGMP levels in rat pituitaries whereas Bicknell *et al* (393) observed no effect of somatostatin on basal or acetylcholine (ACh)-stimulated content of cGMP in bovine pituitaries.

Notwithstanding the several experiments in which somatostatin actions on GH release and cellular cAMP content parallel one another there exist a number of examples of dissociation between these two actions. Stimulation of cAMP accumulation by sodium fluoride and guanylylimidodiphosphate, both which act upon adenylate cyclase, was not affected by somatostatin (390). Exposure of rat pituitary halves to Ba^{++} ion caused an increase of GH release without an effect on cellular cAMP content (386). Somatostatin inhibited the Ba^{++}-stimulated GH release but did not alter pituitary cAMP levels (386). In contrast, somatostatin inhibited cellular cAMP levels in a cloned rat pituitary tumor line, MtT-F4, but did not affect GH secretion (394). ACh stimulated both GH release and cGMP accumulation by bovine pituitaries; somatostatin inhibited ACh's effect on GH release but not on cGMP accumulation (393). In summary, mediation of somatostatin's actions on GH release exclusively by cyclic nucleotides is inconsistent with the

above observations. Alternative mechanisms probably participate in determining somatostatin's action.

b. Calcium ion. Although the presence of the calcium ion is required for pituitary GH release, present studies do not permit a clear answer whether alteration by somatostatin of calcium transport across pituitary cell membranes is important to somatostatin's action. Stimulation of GH release from cultured rat pituitary cells by various secretogogues was dependent on the extracellular calcium ion concentration (385). Somatostatin partially inhibited GH release from rat pituitary cells *in vitro* stimulated by a calcium ionophore (385). Addition of a high extracellular potassium concentration (395,396), combined prostaglandin E_2 and 3-isobutyl-1-methylxanthine (397) or a calcium ionophore (398) to perifused bovine pituitary tissue resulted in an increase of GH release and efflux of $^{45}Ca^{++}$. In each case somatostatin inhibited the release of GH without effect on the $^{45}Ca^{++}$ efflux, an observation which suggests noninvolvement of calcium membrane flux in the biological effects of somatostatin on the anterior pituitary. In contrast, somatostatin inhibited both GH release and $^{45}Ca^{++}$ efflux from bovine pituitaries *in vitro* stimulated by ACh (393). Additional evidence supporting an alteration of calcium flux in somatostatin's actions is that somatostatin reduced the $^{45}Ca^{++}$ uptake into cloned hormone-secreting cells in a basal state (399), and bovine pituitary cells stimulated by high extracellular potassium concentration (396). However, another study observed that somatostatin had no effect upon the potassium-stimulated uptake of $^{45}Ca^{++}$ by bovine pituitary cells (395).

c. Cellular binding sites for somatostatin. Schonbrunn and Tashjian (400) have described cellular binding sites for somatostatin on a cloned rat pituitary tumor cell line, GH_4C_1, that secretes both GH and PRL. The presence of somatostatin receptors on different cell strains correlated well with the capacity of the cells to respond to somatostatin. The dose-response characteristics of somatostatin binding to GH_4C_1 cells and inhibition of GH and PRL secretion were quantitatively similar. Somatostatin has also been reported to bind to bovine anterior pituitary cell membranes but with a considerably lower affinity than to GH_4C_1 cells (401). No comparison was made in this study between the characteristics of somatostatin binding to

the membrane and the biological action of somatostatin on the bovine pituitary. Ogawa *et al* (218,402) have identified and described the physicochemical characteristics of a heat-labile somatostatin binding protein in the cytosol fraction of several rat tissues including the brain and anterior pituitary. Although probably of intracellular origin, the binding protein could represent an easily soluble membrane protein. It has an apparent molecular weight of 80,000 daltons and disulfide linkages are required for its binding to somatostatin. The relationship between the biological action of somatostatin and its binding to the soluble protein remains to be established.

4. Actions on the Nervous System

a. Electrophysiological studies. Renaud and colleagues (403,404) first demonstrated that microiontophoretic application of somatostatin depressed the firing rate of CNS* neurons. Using anesthetized rats and extracellular recordings they observed a depression of discharge frequency of single neurons in the hypothalamic ventromedial nucleus and the cerebral and cerebellar cortices in response to local iontophoretic application of somatostatin. Other characteristics of the neuronal responses were: i. an increase in the spike amplitude (consistent with membrane hyperpolarization), ii. a brief time of onset after initiation of the ejection current, iii. rapid recovery after cessation of the current, and iv. a graded depression of discharge frequency with increasing current. A higher proportion of neurons were responsive to somatostatin in the hypothalamus and cerebral cortex than in the cerebellar cortex. The authors concluded that their results favor a postsynaptic rather than presynaptic site of action.

Other studies have concluded that iontophoretically-applied somatostatin has an excitatory effect on neurons. Ioffe *et al* (405), using conscious rabbits that were habituated to restraint, noted an increase in the discharge frequency of 58 percent of sensorimotor cortical neurons. The latency of onset varied from 1 to 40 seconds and the recovery period after termination of the ejection current was up to 40 seconds, both intervals being longer than those observed by Renaud *et al* (403). Glutamate stimulation of neuronal discharge frequency was potentiated by simultaneous administration of somatostatin. In contrast, Renaud *et al* (403) observed

*(CNS) central nervous system

that somatostatin depressed glutamate-stimulated neuronal firing. Dodd and Kelly (406) used tissue slices of rat hippocampus and recorded intracellularly from CA_1 and CA_2 pyramidal neurons. They observed transmembrane depolarization and an increased frequency of action potentials in response to administration of somatostatin by microiontophoresis, by direct pressure injection, and by local application of droplets. The excitatory effects of somatostatin were comparable to those of glutamate in latency and magnitude. In this study a negative current was employed to discharge the somatostatin at pH 7.0 in contrast to the previous studies (403-405) which had used a positive current of lesser magnitude. Dodd and Kelly did not observe any alteration of neuronal excitability on passage of a positive current. Notwithstanding the different currents used in the microiontophoretic studies, the similar results obtained by Dodd and Kelly using three methods of application of somatostatin speak for the validity of their observations.

Randic and Mitelic (407) observed in the cat that microiontophoretic application of somatostatin depressed the frequency of spinal cord dorsal horn neuron discharges which occurred spontaneously or were activated by noxious stimuli applied to the skin. Dorsal horn neurons activated by non-noxious mechanical stimuli were not affected by somatostatin. The characteristics of the depressant response, rapid onset and recovery associated with an increase in spike amplitude, were comparable to those observed by Renaud *et al* (403). Methionine-enkephalin applied microiontophoretically produced similar results to somatostatin, although the opiate receptor antagonist naloxone blocked the depression of the firing rate after methionine-enkephalin but not after somatostatin. On the basis of these results and immunohistochemical evidence that somatostatin is localized in primary afferent neurons (175), the authors suggested that somatostatin may participate in the modulation of pain sensation.

Koranyi *et al* (408) have accumulated evidence for a depressant effect of somatostatin on neuronal discharges in the mesencephalic reticular formation of the rat. Intraperitoneal injection of somatostatin increased the time interval between spike discharges in the brain stem reticular formation but only minimally affected the interspike interval in the

hippocampus. The authors concluded that somatostatin decreased neuronal activation in the reticular formation but could not rule out the possibility that somatostatin could also depress neuronal activity in the hippocampus. Such an effect could have been masked by a competing tendency toward hippocampal activation occurring physiologically as a consequence of decreased reticular formation activation. The interpretation of these data are made difficult by lack of any conclusive evidence that somatostatin crosses the blood-brain barrier.

That somatostatin perturbs the electrophysiological status of neurons is undeniable; what is uncertain at present is how to interpret these effects in terms of a possible physiological function for somatostatin in the nervous system. The varying results obtained in studies of the brain may be due to a number of factors, including differences in species, anatomical regions studied, and experimental methods. Two limitations apply to microiontophoresis as an experimental model. 1. The specificity of the neuronal response has not been established. Renaud *et al* (404) have demonstrated that a relatively large number of putative neurotransmitters and peptides depress the firing rate of hypothalamic neurons. Unfortunately, no specific somatostatin receptor blocking agent is currently available to assist in the study of this question. Control studies using analogs of somatostatin would be pertinent. 2. Dose response relationships between electrophysiological events and somatostatin release from the microiontophoretic barrels have not been defined. Presently available techniques do not permit simultaneous quantitation of peptide release and determination of biological effect at a cellular level.

b. Behavioral studies. Administration of somatostatin into the CNS of conscious rats produces a characteristic spectrum of behavior. Havlicek and colleagues (409) observed an initial excitation with reduction of both slow wave sleep (SWS) and rapid eye movement (REM) sleep after icv infusion of 10 µg of somatostatin. This initial effect progressed to motor incoordination and rigidity of limb extensors in a majority of animals; one third developed tonic-clonic seizures. Contraction of limb extensors resulted in rotation of the animal unidirectionally on the longitudinal (body) axis, which was termed "barrel rotation" by Cohn and Cohn

(410) and which has been observed by other groups (411-413). Administration of the muscarinic blockers atropine (410) and trihexyphenidyl (413) inhibited "barrel rotation" but haloperidol, apomorphine, reserpine, or amphetamine did not, suggesting involvement of a cholinergic mechanism.

Direct stereotaxic injection of somatostatin into selected brain regions of the rat , including the amygdala, hippocampus, neostriatum and cerebral cortex, produced similar behavioral effects (414-417). A dose of 0.01 to 0.1 µg caused behavioral activation, stereotyped behavior, and slight motor incoordination. With increased doses of 1.0 to 10 µg a sequence of motor disturbances ranging from incoordination, muscle tremors and rigidity, to tonic-clonic seizures resulted. Sleep disturbances characterized by a reduction of REM and deep SWS occurred with somatostatin administration to each brain region. Rezek *et al* (418) commented that several of the behavioral effects of somatostatin were similar to those of morphine. It is difficult to interpret the significance of these responses in relation to specific regional brain effects. Since the doses exceeded local concentrations of somatostatin it is possible that the effects were mediated via extracellular or CSF distribution of the peptide over a considerable distance.

c. Interaction with compounds active on the CNS. Relatively few studies have investigated the interactions of somatostatin with agents known to act on the CNS. As part of a screening study of the systemic pharmacological effects of somatostatin, Plotnikoff *et al* (419) observed that this peptide potentiated the action of L-dopa in inducing hyperactivity. Brown and Vale (420) reported that intravenous somatostatin (1 mg/kg) reduced by 30 percent the dose of phenobarbital which caused death in 50 percent of rats studied (LD_{50}). In addition, somatostatin decreased the duration of seizures induced by strychnine and increased the LD_{50} of strychnine by 21 percent. These authors postulated a CNS depressant effect of somatostatin. To study whether *endogenous* somatostatin contributes to the responses of rats to strychnine and pentobarbital Chihara *et al* (421) administered antisomatostatin gamma-globulin icv. Antisomatostatin globulin reduced the duration of strychnine-induced seizures and increased the LD_{50} of strychnine but resulted in an increase in the LD_{50} of pentobarbital.

The studies of Brown and Vale and Chihara *et al* are in agreement in concluding that exogenously-administered and endogenous somatostatin potentiate the CNS depressant effect of pentobarbital. However, the studies disagree concerning the effects of somatostatin on strychnine's action; exogenous somatostatin reduced the strychnine response but endogenous somatostatin was interpreted to potentiate strychnine's action. This discrepancy has been explained by the authors as possibly due to differences in the doses of somatostatin involved, low in the case of endogenous somatostatin and high in the case of exogenous somatostatin. The studies may not be strictly comparable because of the use of different routes of administration of somatostatin or globulin. The passage of somatostatin into the CNS has not been studied; whether modifications to the molecule or metabolic alterations in the CNS accompany such entry is unknown.

Brown *et al* (422) have proposed that somatostatin may exert a modulatory effect on the CNS regulation of blood glucose concentration. Hyperglycemia and hyperglucagonemia induced by icv administration of bombesin to rats was inhibited by simultaneous icv injection of somatostatin. The possibility that somatostatin could leak out of the CSF into the peripheral circulation to act directly on the pancreas was ruled out by the use of the analog, des-amino acid^{1,2,4,5,12,13}-[D-Trp^{8}]somatostatin, that has no action on glucagon release when given systemically but which, when given icv, inhibited bombesin-stimulated hyperglycemia and hyperglucagonemia. Similarly, hyperglycemia caused by surgical stress and icv injection of β-endorphin or carbachol was reduced by icv somatostatin. Because bombesin's action on blood glucose was dependent on intact adrenal glands the authors concluded that bombesin acted within the brain to increase sympathetic outflow and adrenal secretion which subsequently resulted in hyperglycemia. Somatostatin inhibited bombesin-induced hyperglycemia probably *via* a CNS mechanism to reduce adrenal epinephrine secretion.

d. Interactions with the opiate receptor. Somatostatin displays a relatively weak interaction with the opiate receptor. Cox *et al* (423) first observed that somatostatin was active in the guinea pig ileum bioassay of opiate agonist properties. Somatostatin also inhibited electrically-induced contractions of the mouse vas deferens, a tissue which responds

in a similar manner to opiates, although somatostatin's action was interpreted not to be mediated by the opiate receptor because naloxone did not block the response to somatostatin (424). Terenius (425) determined that somatostatin inhibited binding of ^{3}H-naltrexone and ^{3}H-dihydromorphine to an opiate receptor preparation consisting of synaptic plasma membranes from rat brain. Based on the comparative competition of somatostatin with these two opiate ligands he concluded that somatostatin has partial agonist-antagonist properties. In contrast, Pugsley and Lippman (426) concluded that somatostatin possesses purely agonistic properties based on inhibition of ^{3}H-naloxone binding to a rat brain homogenate preparation. The affinity of somatostatin for the opiate receptor was reduced by 16 fold when sodium ion was added to the incubation medium, a result interpreted to be consistent with pure agonistic activity. The concentration of somatostatin which inhibited the specific binding of ^{3}H-naloxone to the opiate receptor by 50 percent was 5.3×10^{-6} M, a concentration much higher than that required for the inhibition of GH release from cultured pituitary cells or somatostatin binding to pituitary receptors (400). Two synthetic analogs of somatostatin containing amino acids found within the disulfide ring of the molecule demonstrated greater affinity for the opiate receptor. Somatostatin administered icv to rats had an analgesic effect as assessed by the latency of tail flick in response to heat (418). Pretreatment with naloxone blocked the analgesic effect. Kastin *et al* (411) noted that somatostatin given icv to rats antagonized the behavioral effects of previously-administered endorphin including sedation, rigidity, and analgesia but not hypothermia.

e. Neurochemical effects

Somatostatin administered icv to rats increased L-dopa concentrations in the limbic forebrain, corpus striatum, cerebral cortex, diencephalon, and brainstem but increased 5-hydroxytryptamine levels only in the limbic forebrain, corpus striatum and brainstem (412). The effect of somatostatin on the rate of metabolism of monoamines was studied by prior administration of a synthesis inhibitor. Subsequent injection of somatostatin accelerated the rate of decline of dopamine and 5-hydroxytryptamine levels but not norepinephrine levels. The authors suggested that somatostatin increased the rate of synthesis and utilization of brain monoamines.

Somatostatin given icv to rats decreased the content of ACh in parietal cortex but not frontal cortex, striatum, hippocampus, diencephalon or brainstem (413). In addition, somatostatin increased the turnover rate of ACh in the diencephalon, brainstem, and hippocampus (413,427). Local injection of somatostatin into the septal area did not change ACh content or turnover rate in the hippocampus, which has a septal-hippocampal cholinergic input (413). Surgical transection of afferent projections to the hippocampus did not prevent somatostatin's stimulatory action on ACh turnover rate in the hippocampus (427). These observations suggest that somatostatin's effect on hippocampal ACh turnover occurs locally and not indirectly *via* neuronal projections to the hippocampus.

A possible effect of somatostatin on CNS calcium transport was suggested by the studies of Tan *et al* (428). Somatostatin (100 µg/ml) enhanced the glutamate-stimulated uptake of $^{45}Ca^{++}$ into synaptosomes, although somatostatin alone had no effect on $^{45}Ca^{++}$ uptake. In addition, somatostatin inhibited the release of $^{45}Ca^{++}$ from synaptosomes probably through an action on a rapidly-releasable pool. Nemeth and Cooper (429) have cautioned about the interpretation of the action of high concentrations of somatostatin on biological systems. They observed that 100 µg/ml somatostatin stimulated ACh release from rat hippocampal synaptosomes. Because this effect was associated with release of the cytoplasmic enzyme lactic dehydrogenase and was not calcium dependent the authors concluded it was due to a nonspecific action of somatostatin, perhaps by damaging the plasma membrane.

Cyclic AMP levels in the rat hippocampus and caudate/putamen were increased by icv injection of somatostatin, an effect inhibited by the β-adrenergic blocker, sotalol (430). Based on bioassay data, Goldstein and Pavel (431) concluded that intracarotid injection of somatostatin caused release of vasotocin into the CSF of cats.

f. Effects on the peripheral nervous system. Three groups (432-434) have shown that somatostatin decreased the amplitude of contractions of strips of guinea pig ileum induced by electrical field stimulation. Somatostatin did not inhibit contractions due to exogenous administration of ACh or carbachol; naloxone did not modify the response to somatostatin.

Somatostatin also reduced the contractions caused by field stimulation of the rat vas deferens and the rabbit ear artery, tissues sensitive to adrenergic agents (433). Somatostatin did not affect contractions stimulated by exogenously administered norepinephrine. These groups have concluded that somatostatin acts through inhibition of neurotransmitter release. Williams and North (435) on the evidence of electrophysiological studies of ganglion cells of the guinea pig myenteric plexus maintained in a tissue bath concluded that somatostatin has a direct inhibitory action on neuronal firing. Blockade of synaptic transmission did not affect the inhibitory action of somatostatin, suggesting that somatostatin does not act by modification of neurotransmitter release. Naloxone did not block the inhibitory effect of somatostatin, indicating noninvolvement of the opiate receptor.

g. Effect on neurological disorders. A two hour intravenous infusion of somatostatin to patients with extrapyramidal disorders and electroencephalographic abnormalities produced no change in the neurological signs and symptoms nor the electroencephalographic patterns (436).

5. Release from Nervous Tissue

Although somatostatin in the ME almost certainly participates in the regulation of GH secretion, its function in other parts of the nervous system is not yet established. In summary, evidence that somatostatin could function as a neurotransmitter includes: i. the selective distribution of somatostatin in anatomic pathways, as for example, in primary afferent neurons, ii. immunohistochemical localization in nerve terminals and secretory granules, iii. localization by RIA predominantly in synaptosomes, suggesting presence in nerve terminals, iv. alteration of animal behavior after exogenous administration and v. influences on electrophysiological properties of neurons.

An important requirement for a candidate neurotransmitter is that it be released from nervous tissue. Several studies have used a variety of *in vitro* experimental preparations to study IRS release, including i. incubated rat neurohypophysis (437), ii. incubated rat hypothalamic synaptosomes (438), iii. perifused rat hypothalamic and amygdala slices (439),

iv. incubated rat hypothalamic blocks (440), v. perifused rat hypothalamic blocks (441), vi. incubated rat cerebral cortical slices (442), vii. incubated rat hypothalamic slices (443), viii. incubated rat ME fragments (444). In all except the final study listed above the release of IRS was shown to be calcium dependent and stimulated by membrane depolarization using high extracellular potassium concentration. Other agents that have stimulated IRS release include: i. dopamine in incubated rat hypothalamic synaptosomes (438) and rat ME fragments (444), ii. a calcium ionophore (440), rat GH (445), neurotensin and substance P (446) using incubated rat hypothalamic blocks, and iii. norepinephrine in incubated rat ME fragments (444). A large number of neurotransmitters and neurotransmitter candidates tested failed to increase IRS release from perifused rat hypothalamus (441). Vasoactive intestinal polypeptide has inhibited IRS release from incubated rat hypothalamic slices (443).

Interpretation of studies of IRS release into rat hypophysial portal vessels (214,215,447,448) is subject to the reservations described above concerning the validity of RIAs of blood IRS. Using this *in vivo* model Chihara *et al* (447) have reported that icv injection of dopamine, norepinephrine and ACh to urethane-anesthetized rats and dopamine to althesin-anesthetized rats increased IRS release into hypophysial portal blood. Electrical stimulation of the preoptic area of rats increased IRS release whereas electrical stimulation of the ventromedial hypothalamus did not (448). These latter observations are consistent with the high number of IRS-containing neurons in the preoptic area that project to the ME.

There are several deficiencies in our knowledge about somatostatin in nervous tissue that signal caution with respect to considering that somatostatin functions as a neurotransmitter. The mechanism of somatostatin biosynthesis and the characteristics of somatostatin receptors in nervous tissue are unknown. Release of somatostatin from nerves upon stimulation of afferents and correlation of endogenous release and exogenous application with biological effects have not yet been studied. Apart from microiontophoretic studies, little data has been reported concerning events in the recipient cell which accompany somatostatin administration or release. Specific pharmacological antagonists, agonists, synthesis inhibitors or

receptor blocking agents comparable to those used for study of monoaminergic or cholinergic systems are presently not available for somatostatin.

RATIONALE FOR STUDIES UNDERTAKEN

In describing the rationale for undertaking these studies I must attempt to assume the perspective of the state of knowledge about somatostatin that was available at the beginning of these studies. In mid 1976 only one published paper describing a RIA for somatostatin was available (68). The anatomical distribution of IRS had been described but little information was available about the nature of endogenous IRS. In order to undertake study of endogenous IRS in the nervous system we initially developed a RIA using a sheep as the source of antiserum, in part to obtain a large quantity of antiserum. A considerable portion of this thesis is concerned with the characteristics and validation of the RIA. For instance, determination of the site on the somatostatin molecule to which the antiserum binds allows us to use the RIA as a molecular probe for particular IRS determinants in tissue or body fluids.

A bioassay of GH release inhibiting activity was used an an independent indicator of the presence of somatostatin-like activity in tissues. The concomitant observation of GH release inhibiting activity lends validity to demonstrations of IRS made by RIA and, in the case of chromatographically heterogeneous forms of IRS, could indicate the extent to which they could be biologically active.

An additional use of the SS-AS when coupled to an insoluble matrix was as an affinity chromatography ligand for endogenous IRS. Affinity chromatography of somatostatin had not previously been attempted at the time this work was begun and therefore attention was given to several methodological issues as described in the experimental section. Affinity chromatography served mainly to concentrate and partially purify IRS from several brain regions and plasma. Another initial motivating factor was that removal of IRS by affinity chromatography might prove a useful technique for future study of growth hormone releasing factors without the potential interference by somatostatin. Affinity chromatography was used to demonstrate the presence of IRS in plasma. No previously reported study at that time had identified IRS in blood.

RIA, bioassay and affinity chromatography techniques were used to demonstrate the presence of somatostatin-like material in the rat and human retina,

a neural tissue that had not previously been shown to contain somatostatin. The retina is of particular interest as a potential model tissue for the future study of somatostatin action on the nervous system. The anatomy, pharmacology and physiology of the retina have been extensively studied by others and provide a wealth of background information that may assist in the investigation of somatostatin function in the retina.

Very little data on the chromatographic behavior and biological activity of hypothalamic or extrahypothalamic IRS was available at the start of this work. Again the techniques of RIA, bioassay and affinity chromatography were applied to the study of this question. Selected anatomical regions were studied for particular reasons: i. ME because it contained predominantly nerve terminals, ii. anterior hypothalamic-preoptic area because it contained a relatively high concentration of neuronal cell bodies positive for IRS by immunohistochemistry, and iii. amygdala and cerebral cortex as representatives of extrahypothalamic nervous tissues. We were particularly interested whether IRS from these different regions would exhibit similar or different chromatographic and biological properties. Because the ME content of IRS is dependent upon the integrity of anterior hypothalamic-preoptic area connections to the ME we wondered whether IRS in the anterior hypothalamic-preoptic area, where presumably the IRS was synthesized, differed chromatographically and biologically from IRS in the ME, a site to which IRS may have been transported down axons from the anterior hypothalamic-preoptic area. Forms of IRS with heterogeneous chromatographic and biological properties could represent: i. immunologically cross reactive materials unrelated to somatostatin except in sharing common antigenic determinants, ii. large molecular weight biosynthetic precursors of somatostatin, iii. species of somatostatin that were chemically modified in a manner specific to a particular anatomical site, and iv. degradation products of somatostatin. These studies may yield clues to whether somatostatin biosynthesis or metabolism proceeds by identical mechanisms in various tissues or whether particular mechanisms and biological controls of biosynthesis and metabolism exist in different tissues that may modify the expression of somatostatin's biological action in a tissue-specific manner.

EXPERIMENTAL

A. RIA FOR SOMATOSTATIN

1. Materials and Methods

a. Materials. The sources of the peptides used are as follows: somatostatin for immunization (lot AY 24,910), Dr. H. Immer and M. Götz, Ayerst Laboratories; somatostatin for RIA standard (lot 14-206-60), Dr. J. Rivier, Salk Institute (La Jolla California); [Tyr^1]somatostatin, Dr. J. Rivier (lot 35-242-30) and Ayerst Laboratories (lot Ay 25,511); N-Tyr-somatostatin (lot 9185), Bachem Inc.; analogs of somatostatin indicated in Table 9, Dr. J. Rivier; analogs of somatostatin indicated in Table 10, Ayerst Laboratories; glucagon, gastrin, secretin, and gastric inhibitory polypeptide, Dr. J. Dupre, University of Western Ontario (London, Ontario); porcine insulin, Connaught Laboratories (Toronto, Ontario); substance P, Dr. S. Leeman, Harvard Medical School (Boston, Massachusetts); oxytocin, Peninsula Laboratories; vasopressin and rat neurophysin 1, Drs. M.J. Brownstein and H. Gainer, National Institutes of Health, U.S.A.; melanocyte stimulating hormone-release inhibiting factor (lot AY 24,192), Ayerst Laboratories; TRH and LHRH, (lots 21-34-95 and 14-136-30, respectively), Dr. J. Rivier; rat GH, rat LH, rat PRL, and rat TSH (reference preparations GH-RP-1, LH-13, PRL-RP-1, and TSH-RP-1, respectively), National Institutes of Arthritis, Metabolism and Digestive Diseases, U.S.A.

The sources of the following reagents are: human serum albumin (HSA) (Cohn fraction V), HSA (globulin free, crystallized), bovine serum albumin (BSA)(RIA grade), methylated BSA, chicken egg albumin (grade V, electrophoretic purity), and gelatin (type III, calf skin), Sigma Chemical Co.; [^{125}I]NaI (100 mCi/ml, 1 Ci=3.7x10^{10} becquerels), Amersham/Searle; carboxymethyl cellulose (CM-52), Whatman, Inc.; Freund's complete and incomplete adjuvants, Difco Laboratories; sheep gamma globulin (lot 9661), ICN Pharmaceuticals; sheep serum, Pel Freeze Biologicals, Inc.; Rabbit antiserum against sheep gamma globulin, Antibodies Incorporated; fluorescamine (Fluram), Hoffman-LaRoche.

b. Induction of antisera. Antisera to somatostatin were induced in two adult sheep by use of a method that had previously proven successful in our laboratory for the development of a SS-AS in rabbits (73). Seven mg of synthetic somatostatin were dissolved in 2.5 ml of 0.15 mol/l NaCl and mixed with 2 ml distilled H_2O containing 1 mg methylated BSA. The solution was emulsified with an equal volume of Freund's adjuvant. Four ml of the emulsion were injected intradermally at multiple sites into each of two adult sheep (one male Dorset type, 54 kg, and one female Suffolk type, 41 kg). The sheep sera were tested for their ability to bind [^{125}I-Tyr^1] somatostatin four weeks after the first injection and at two to three week intervals thereafter. Booster injections consisted of the same emulsion except that Freund's incomplete adjuvant was used. These were given intramuscularly at intervals of two to three weeks. Both sheep had generated antisera within a period of 4-5 months. The SS-AS produced by the male sheep subsequently proved to have a higher titer and gave a more sensitive standard curve in the RIA.

c. Radioiodination of [Tyr^1]somatostatin. Because somatostatin does not contain an amino acid suitable for iodination the analog [Tyr^1]somatostatin was used to generate a radioiodinated antigen for RIA. [Tyr^1]somatostatin was radioiodinated by a modification of the method of Greenwood *et al* (449). To 25 µl 0.5 mol/l sodium phosphate buffer, pH 7.4, were added 100 µl 5.78×10^{-5} mol/l (10 µg) [Tyr^1]somatostatin in 0.002 mol/l ammonium acetate buffer, pH 4.6. One to two mCi of [^{125}I]NaI (100 mCi/ml) was added, followed by 25 µl freshly prepared 6.59×10^{-4} mol/l chloramine-T (0.15 mg/ml) dissolved in 0.05 mol/l sodium phosphate buffer, pH 7.4. After a reaction time of 30-40s at room temperature, 50 µl 1.31×10^{-3} mol/l sodium metabisulfite (0.25 mg/ml) dissolved in 0.05 mol/l sodium phosphate buffer, pH 7.4, were added. This was followed, after an additional 10s, by 100 µl 10% (wt/vol) HSA (Cohn fraction V) dissolved in 0.1 mol/l sodium phosphate buffer, pH 7.4. The radioiodinated product was purified by ion exchange chromatography (Figure 1) on a column of carboxymethyl-cellulose (CM-52), by a previously reported method (68,73). The first sharp peak corresponds to the elution of unreacted radioactive iodide. The radioiodinated product in the second peak bound to the SS-AS.

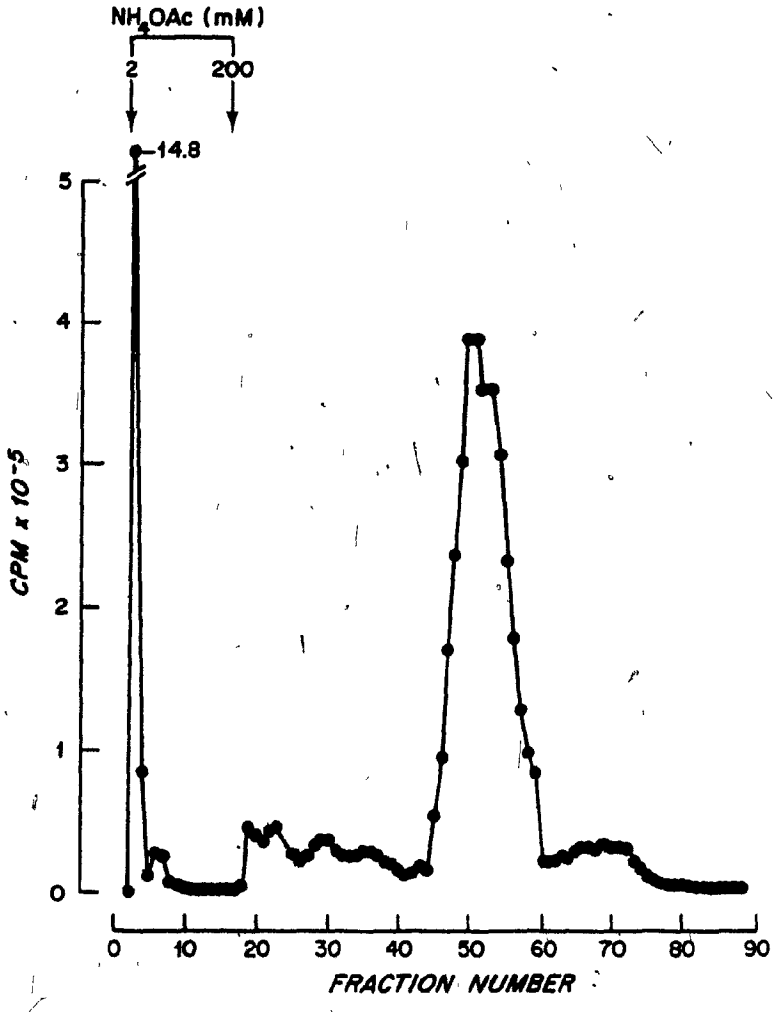


Figure 1. Ion exchange chromatography of [^{125}I-Tyr^{1}]somatostatin. A 0.7 × 15 cm column was used. Sequential elutions with 2 and 200 mmol/l ammonium acetate buffers are indicated. The fraction size was 1.7 ml.

Incubations of [^{125}I-Tyr^{1}]somatostatin from the second peak in serially increasing mass under the conditions of the RIA resulted in a curve of binding of radiolabel to the antiserum which was parallel to the radiolabel binding curve obtained in the presence of synthetic somatostatin (Figure 2). The specific activity of the [^{125}I-Tyr^{1}]somatostatin calculated from the binding data was 261 µCi/µg. N-Tyr-somatostatin was iodinated by the identical procedure described above and produced on similar elution profile as [^{125}I-Tyr^{1}]somatostatin on ion exchange chromatography (data not shown).

In order to make a rational decision about the composition of the RIA buffer, the stability of [^{125}I-Tyr^{1}]somatostatin was studied in the presence of different proteins and EDTA concentration. Approximately 4 x 10^{5} counts per minute (cpm) [^{125}I-Tyr^{1}]somatostatin were incubated for 24 h and 7 days in 0.4 ml buffer consisting of 0.1 mol/l sodium phosphate (pH 7.2), 0.05 mol/l NaCl, 0.02% (wt/vol) NaN_3 and the protein and EDTA solutions shown in tables 7 and 8. After the incubation period approximately 10,000 cpm were removed for immunoprecipitation using 1:10,000 final concentration of SS-AS and addition of second antibody as described below for the routine RIAs. Tables 7 and 8 indicate that purified BSA resulted in less inhibition of [^{125}I-Tyr^{1}]somatostatin binding to SS-AS than other proteins. A similar study by Patel and Reichlin (450) indicated that the inhibition of [^{125}I-Tyr^{1}]somatostatin binding by serum was prevented by boiling, therefore suggesting that it was due to enzymatic activity. Tables 7 and 8 demonstrate that use of 0.01 or 0.25 mol/l EDTA partially prevented the protein-related inhibition of binding. The tendency of somatostatin to adhere to glass necessitated the use of a nonspecific protein in the RIA buffer. The routine buffer used for RIA was 0.1 mol/l sodium phosphate (pH 7.2), 0.05 mol/l NaCl, 0.01 mol/l EDTA, 0.02% (wt/vol) NaN_3, 0.1% (wt/vol) HSA (Cohn fraction V).

The stability of [^{125}I-Tyr^{1}]somatostatin and ^{125}I-labelled-N-Tyr-somatostatin was determined after periods of storage at 4°and -30°C (Figures 3 and 4). The percentage binding of labelled analog was determined using a 1/10,000 final dilution of SS-AS under routine RIA conditions described below. It is clear that storage at 4°C in the absence of added 0.1% (wt/vol) HSA (crystalline) resulted in marked deterioration of binding

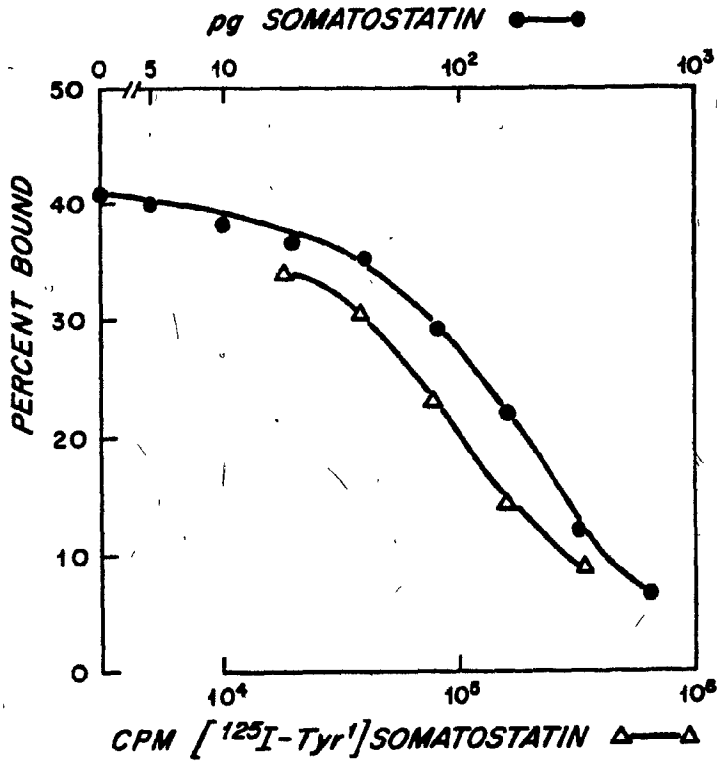


Figure 2. Binding of [^{125}I-Tyr^1]somatostatin to antiserum as a function of the mass of somatostatin or counts per minute (cpm) [^{125}I-Tyr^1]somatostatin per RIA tube. Percent bound equals cpm [^{125}I-Tyr^1]somatostatin specifically bound to antiserum divided by total cpm [^{125}I-Tyr^1]somatostatin (minus cpm nonspecifically bound) × 100.

Table 7. Effect of incubation conditions on specific binding of [^{125}I-Tyr^{1}] somatostatin to antiserum: 24 h incubation

Protein	Concentration %	Specific Binding %		
		EDTA 0 mol/l	EDTA 0.01 mol/l	EDTA 0.025 mol/l
Bovine serum albumin (RIA grade)	0.1 wt/vol	50.2	49.3	48.5
	0.5 "	50.5	51.0	52.4
Human serum albumin (Crystalline)	0.1 "	42.4	49.1	48.7
	0.5 "	46.0	42.7	50.8
Human serum albumin (Cohn fraction V)	0.1 "	45.1	51.8	51.8
	0.5 "	34.8	46.2	48.1
Egg albumin (chicken)	0.1 "	42.6	56.0	47.8
	0.5 "	35.2	38.0	44.5
Gelatin (calf skin)	0.1 "	40.5	43.0	33.6
Sheep serum	10 vol/vol	1.7	3.3	5.1
	1 "	24.1	32.5	34.3
	0.1 "	43.1	68.1	47.9

Table 8. Effect of incubation conditions on specific binding of [^{125}I-Tyr^{1}] somatostatin to antiserum: 7 day incubation

Protein	Concentration %	Specific binding %		
		EDTA 0 mol/l	EDTA 0.01 mol/l	EDTA 0.025 mol/l
Bovine serum albumin (RIA grade)	0.1 wt/vol	51.6	57.5	56.0
	0.5 "	49.8	57.3	57.4
Human serum albumin (crystalline)	0.1 "	43.0	48.4	45.9
	0.5 "	35.6	39.5	39.7
Human serum albumin (Cohn fraction V)	0.1 "	29.4	56.7	49.7
	0.5 "	10.0	39.3	40.0
Egg albumin (chicken)	0.1 "	33.1	54.0	55.8
	0.5 "	11.1	50.6	45.9
Gelatin (calf skin)	0.1 "	34.3	47.6	52.0
Sheep serum	10 vol/vol	0.5	0.0	0.2
	1 "	2.0	16.4	17.1
	0.1 "	30.0	45.8	52.5

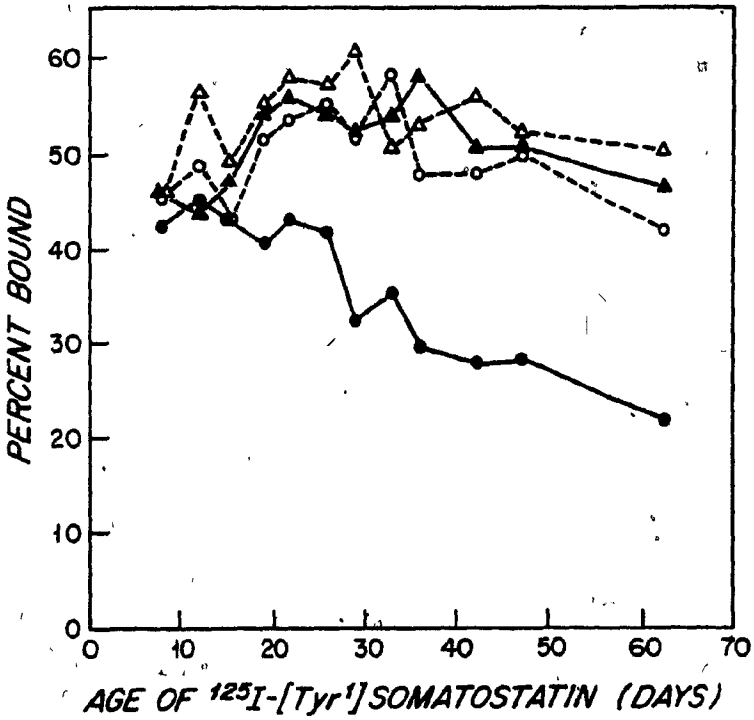


Figure 3. Binding of [^{125}I-Tyr^{1}]somatostatin to antiserum as a function of days of storage of [^{125}I-Tyr^{1}]somatostatin. Percent bound is defined in Figure 2. Storage conditions: ● 4°C, ▲ 4°C with 0.1% HSA (crystalline) added, ○ -30°C, △ -30°C with 0.1% HSA (crystalline) added.

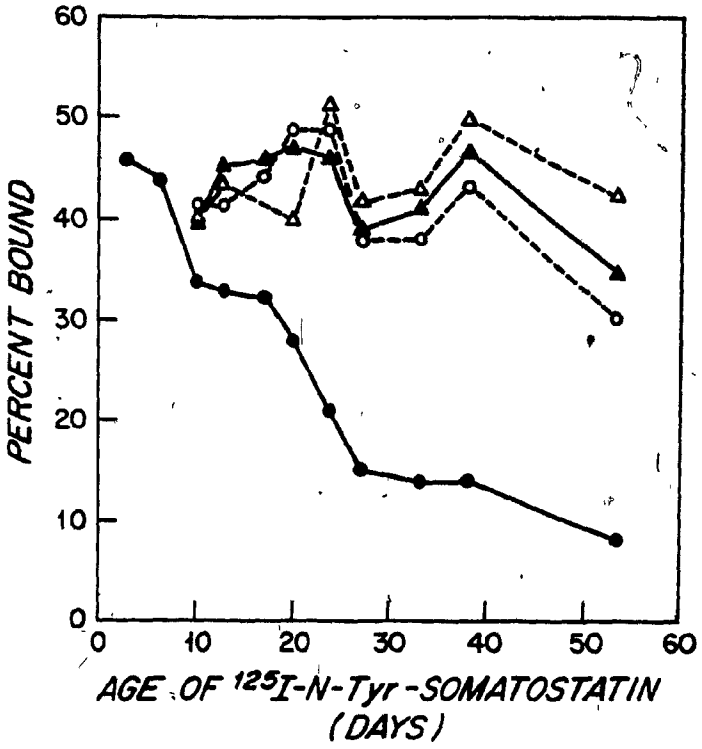


Figure 4. Binding of ^{125}I-labelled N-Tyr-somatostatin to antiserum as a function of days of storage of ^{125}I-labelled N-Tyr-somatostatin. Percent bound is defined in Figure 2. The symbols are defined in Figure 3.

over time. Based on these results storage of the labelled analog below 0°C is recommended. The concentration of somatostatin in the RIA standard curve required to inhibit the specific binding of [^{125}I-Tyr^{1}]somatostatin to antiserum by 50% (ED_{50}) remained relatively stable over time under different storage conditions (Figure 5).

d. RIA. RIA was routinely performed using a 1:20,000 or 1:25,000 final dilution of sheep B (male) antiserum and 10,000 to 12,000 cpm [^{125}I-Tyr^{1}]somatostatin in a final volume of 400 µl in 10 mm diameter x 75 mm length glass tubes. Standard somatostatin and unknown samples were added in a volume of 100 µl and incubated for 20-24 h at 4°C. Then a portion of rabbit or horse antiserum raised against sheep gamma globulin was added at a dilution determined experimentally to yield an easily visualized pellet and a satisfactory precipitation of the SS-AS. In most cases the final dilutions used were: normal sheep serum 1:1000 and either rabbit antiserum against sheep gamma globulin 1:50 or horse antiserum against sheep gamma globulin 1:40. Following an additional 16-20 h incubation at 4°C the tubes were centrifuged at 2000 × *g* and 4°C for 30 min. The radioactivity in the pellet was determined by a gamma scintillation counter, for most experiments an LKB Rack-Gamma with a counting efficiency of 76.2%. Non-specific binding of [^{125}I-Tyr^{1}]somatostatin in the RIA was 3 to 6%.

The horse antiserum against sheep gamma globulin was raised in a 2 year old horse by subcutaneous injection at two sites of an emulsion of 10 mg sheep gamma globulin, 2 ml of 0.01 mol/l sodium phosphate buffer (pH 7.4), 0.15 mol/l NaCl and 2 ml of complete Freund's adjuvant. A low titer of SS-AS was present five weeks after the primary immunization but the titer increased after booster injections of the above emulsion except using incomplete instead of complete Freund's adjuvant.*

e. Extraction of IRS from tissue. Adult male Charles River CD rats were housed in a temperature and humidity controlled room with lights

*Done in collaboration with Dr. Robert Benoit.

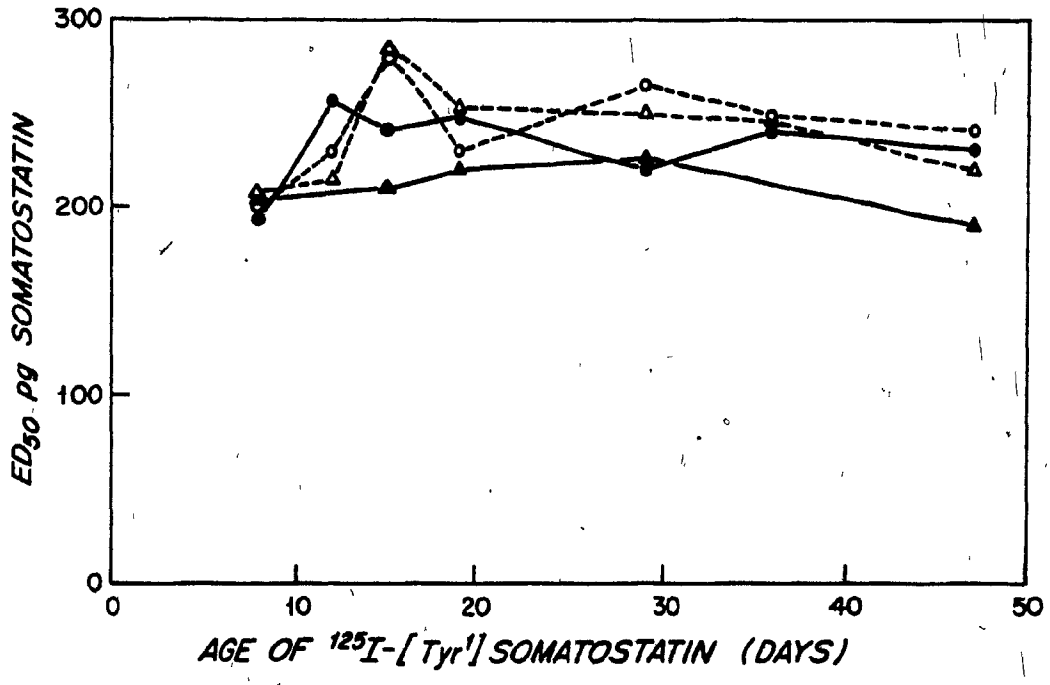


Figure 5. Mass of somatostatin per tube required to displace 50% of [^{125}I-Tyr^1]somatostatin in the RIA (ED_{50}) as a function of days of storage of [^{125}I-Tyr^1]somatostatin. The final dilution of antiserum was 1:10,000. The symbols are defined in Figure 3.

on from 0600 to 1800 h and were allowed free access to Purina Rat Chow and tap water. The rats were killed by decapitation and tissue fragments were dissected over crushed ice. The tissue fragments obtained were: 1. hypothalamus (anterior boundary-optic chiasm, posterior boundary-mammillary bodies, lateral boundaries-hypothalamic sulci, depth 1-2 mm), 2. anterior hypothalamic-preoptic area (a triangular piece of tissue with its base being the anterior limit of the above fragment, depth 1-2 mm), 3. olfactory bulbs, 4. amygdala (a cubic fragment of grey matter with similar anterior and posterior boundaries as the hypothalamic fragments), 5. cerebral cortex (slices of grey matter from the parietal region), 6. brainstem (pons and medulla), 7. cerebellum, 8. cervical spinal cord, 9. pancreas (whole or sectioned parallel to its long axis), 10. stomach (whole stomach or antrum), 11. duodenum (proximal portion), 12. jejunum (15-20 cm from pylorus), 13. colon (descending portion), and 14. liver. The gastrointestinal regions were washed with 0.15 M NaCl. After determination of wet weight the fragments were placed in ice cold extraction solvent, most often 2 mol/l acetic acid or 0.1 mol/l hydrochloric acid (HCl) (tissue wt/vol extraction solvent, <0.1). In some experiments the tubes were placed in a boiling water bath for five min and then allowed to cool. The fragments were homogenized using a glass vessel and rotating Teflon pestle with a clearance of 0.08 to 13 mm. The homogenate was frozen at -20°C overnight and after thawing at room temperature was centrifuged at 2000 × *g* and 4°C for 30 min. The clear supernatant was collected and stored at -20°C. Prior to RIA the extract was diluted in RIA buffer, neutralized using NaOH and phenol red pH indicator, and centrifuged at 2000 × *g* for 10 min to remove a small amount of precipitate that formed on neutralization. The protein content of tissue extracts was determined by a fluorometric method using fluorescamine (451). Bovine serum albumin (RIA grade) served as the reference protein.

2. Results

a. Radioimmunoassay. The specific binding of [^{125}I-Tyr^{1}]somatostatin to the female sheep (A) antiserum was 42.3% at a final antiserum dilution of 1:5000. The mass of somatostatin required to displace 50% of the speci-

fically-bound [^{125}I-Tyr^{1}]somatostatin was 1050 pg/tube. The male sheep (B) antiserum was used for all subsequent studies because its titer and sensitivity were superior to those of the female sheep antiserum.

A Scatchard (452) plot of the binding data obtained under the conditions of the RIA is shown in Fig. 6. The curve is consistent with multiple binding sites for somatostatin or negative cooperativity of the sites at higher concentrations of somatostatin. At lower concentrations of somatostatin the binding data best fit a straight line with an apparent affinity constant of 7.47×10^{9} 1/mol. Figure 7 displays the mean standard displacement curve derived from 13 RIAs. The indicated peptides in concentrations (mass per volume) of 100 times the maximum concentration of somatostatin used for the standard curve did not displace [^{125}I-Tyr^{1}] somatostatin from the sheep antiserum.

The immunological cross-reactivity of several analogs of somatostatin are illustrated in Tables 9 and 10. Alterations of the N and C-terminal portions of the somatostatin molecule did not affect its immunoreactivity greatly but modifications to the central portion of the molecule corresponding to the sequence of amino acids 4-10 reduced its immunoreactivity substantially.*

The minimum detectable concentration of the RIA, defined as the concentration of somatostatin which resulted in binding of [^{125}I-Tyr^{1}] somatostatin to antiserum which was two standard deviations below the mean binding achieved in the absence of somatostatin was 14.3 pg/tube (mean of 16 assays). The within-assay coefficients of variation for samples containing means of 91 and 304 pg/tube somatostatin were respectively 11.0 (seven determinations) and 12.3 (nine determinations). The between assay coefficients of variation of samples containing means of 284,144 and 75 pg/tube somatostatin were 11.5, 13.2 and 13.7 respectively (16 assays).

RIA data in most cases were analysed with a programable desk top calculator (Texas Instruments TI-59) and a program developed by Dr. Keith Hoyte that uses a logit/natural logarithm standard curve calculated by linear regression analysis.

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*Cross reactivity studies shown in Fig. 7 and Tables 9 and 10 were done in collaboration with Drs. J. Epelbaum and P. Brazeau.

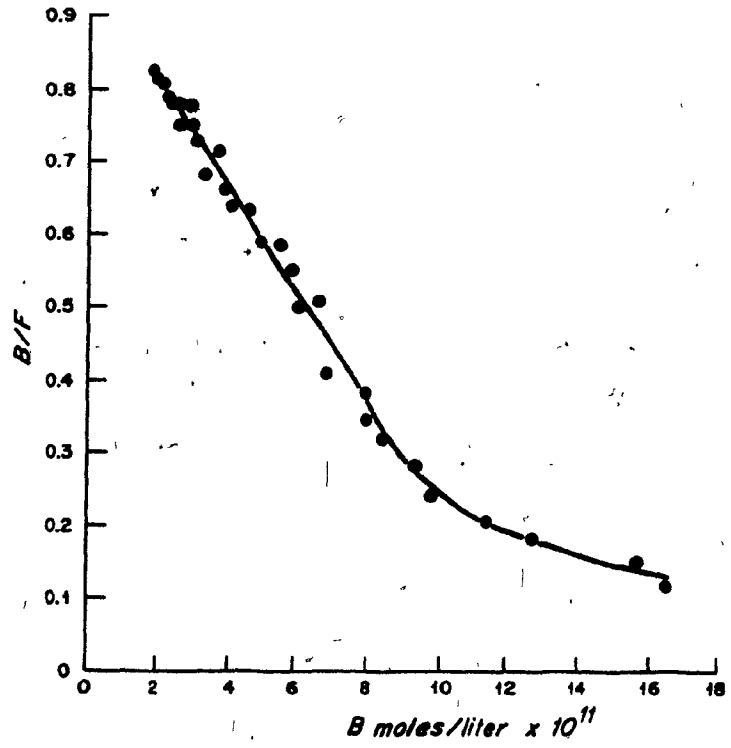


Figure 6. Scatchard plot of binding of somatostatin to antiserum. The experimental conditions were similar to those of the RIA, except that the antiserum was present at a 1:10,000 dilution. B/F, Ratio of bound to free [^{125}I-Tyr^{1}]somatostatin. B on the horizontal axis is the total concentration of [Tyr^{1}]somatostatin and somatostatin bound to antiserum.

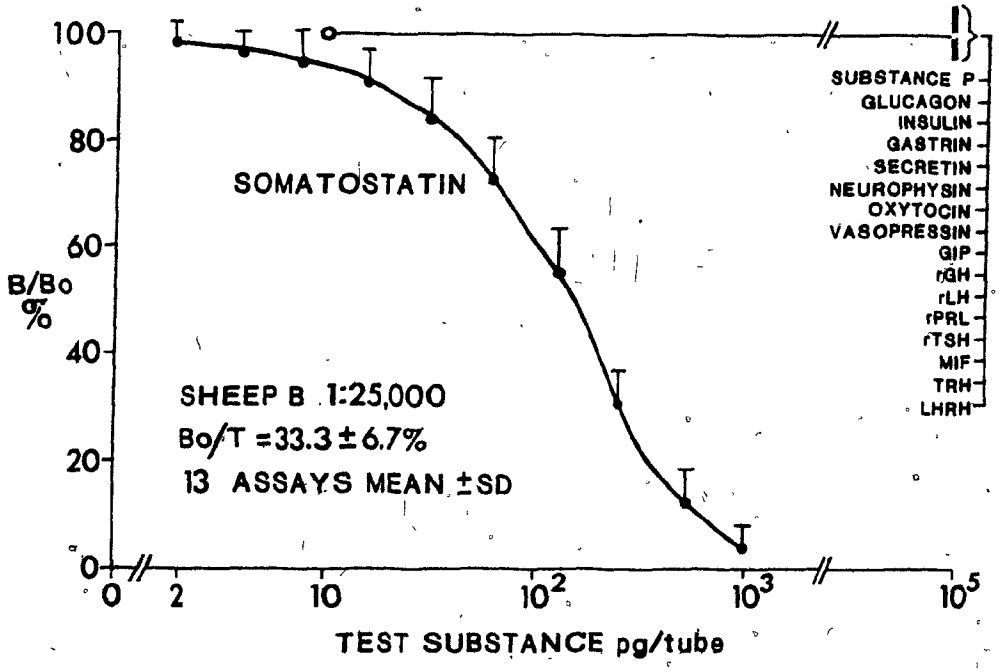


Figure 7. Binding of [^{125}I-Tyr^{1}]somatostatin to antiserum as a function of somatostatin or other peptide concentrations. B/Bo, Ratio of [^{125}I-Tyr^{1}]somatostatin bound in the presence of somatostatin or other peptide to that bound in the absence of synthetic somatostatin. Sheep B refers to the animal whose antiserum was used for these studies. T, Total cpm added to the assay. Each point is the mean of 13 separate assays. The vertical lines indicate the standard deviation (SD). GIP, Gastric inhibitory polypeptide; rGH, rat GH; rLH, rat LH; rPRL, rat PRL; rTSH, rat TSH; MIF, melanocyte stimulating hormone release inhibiting factor.

Table 9.

Immunoreactivity of alanine-substituted analogs of somatostatin:

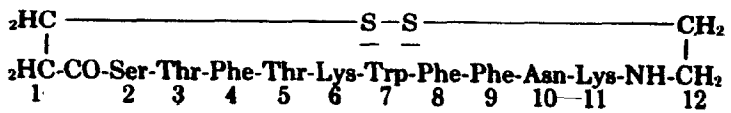
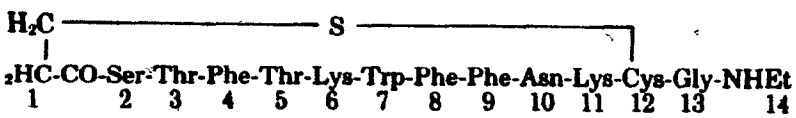
Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Analog	ED_{50} somatostatin/ ED_{50} analog (%)
Somatostatin	100
[Ala^{2}]Somatostatin	175
[Ala^{4}]Somatostatin	1
[Ala^{5}]Somatostatin	11
[Ala^{6}]Somatostatin	1
[Ala^{7}]Somatostatin	1
[Ala^{8}]Somatostatin	1
[Ala^{9}]Somatostatin	21
[Ala^{10}]Somatostatin	18
[Ala^{11}]Somatostatin	66
[Ala^{12}]Somatostatin	61

ED_{50}, Concentration causing 50% displacement of ^{125}I-labeled [Tyr^{1}]somatostatin from antiserum.

Table 10.

Immunoreactivity of analogs of somatostatin

Analog	Δ Analog/Δ somatostatin (%)
Somatostatin	100
[Tyr^1]Somatostatin	105
Gly-Gly-Gly-Somatostatin	101
Leu-Gly-Gly-Somatostatin	98
Leu-Gly-Gly-des-COOH^{14}-Somatostatin	101
des-NH$_2^1$-Somatostatin	93
des-COOH^{14}-Somatostatin	75
des-(NH$_2^1$, COOH^{14})Somatostatin	97
des-(Ala^1, Gly^2, NH$_2^3$)Somatostatin	96
des-(Ala^1, Gly^2, NH$_2^3$, COOH^{14})Somatostatin	108
des-(Ala^1, Gly^2)N^3-Ac-Somatostatin	78
des-Lys^4-Somatostatin	34
des-(Lys^4, Lys^9)Somatostatin	18
	25
Retroenantio isomer of des-(Ala^1, Gly^2, NH$_2^3$, COOH^{14})somatostatin	
	<1
Retroenantio isomer of des-(NH$_2^1$, COOH^{14})somatostatin	

Δ, Displacement of ^{125}I-labeled [Tyr^1]somatostatin from antiserum by somatostatin or an analog of somatostatin, derived from: $\Delta = B_{10} - B_{100,000}/T$, where B_{10} and $B_{100,000}$ are the counts per min of ^{125}I-labeled [Tyr^1]somatostatin bound to antiserum in the presence, respectively, of 10 and 100,000 pg somatostatin or analog. T is the total counts per min.

b. Extraction of IRS from rat tissues. Extraction of rat hypothalamus and cerebral cortex with either 2 mol/l acetic acid or 0.1 mol/l HCl gave similar concentrations of IRS, however extraction of rat pancreas and stomach with 2 mol/l acetic acid yielded significantly higher levels of IRS than extraction with 0.1 mol/l HCl (Table 11). The concentration of IRS in 0.1 mol/l HCl extracts of rat hypothalamus and cerebral cortex declined considerably with prolonged storage at -20°C compared to the IRS concentration in extracts of the same regions stored in 2 mol/l acetic acid (Table 12). Acetic acid extraction was used for the majority of subsequent experiments. Preliminary experiments using a previously described RIA (73) based on a rabbit antiserum raised in our laboratory demonstrated that 2 or 0.2 mol/l acetic acid extracted a greater amount of IRS per mg wet weight from rat hypothalamus, amygdala and cerebral cortex compared to acetone, methanol, or a solution of ethanol-chloroform-H_2O-glacial acetic acid (810:100:90:5). In addition, extraction of rat hypothalamus, amygdala and cerebral cortex by heating the fragments to 100°C for 5 min in 2 mol/l acetic acid did not result in a signficantly higher concentration of IRS than extraction of fragments of the same tissues without heating.

Acetic acid (2 mol/l) extracts of rat hypothalamus, amygdala, cerebral cortex, pancreas, whole stomach, duodenum, jejunum, and colon displaced [^{125}I-Tyr^{1}]somatostatin from antiserum in a fashion parallel to synthetic somatostatin (Figures 8 and 9). The data on the duodenum, jejunum and colon are not shown. The displacement lines were calculated by a nonweighted linear regression analysis using a logit/natural logarithm transformation of the data (453). The 95% confidence limits of the slopes of the displacement lines were determined (454). In all cases the slopes of the displacement lines of the tissue extracts did not differ significantly from the slopes of the displacement lines of synthetic somatostatin when compared by a t-test ($p<0.1$).* This suggests immunological similarity between somatostatin and the tissue extracts.

In order to validate the extraction procedure for somatostatin and to determine whether the presence of a tissue extract would interfere with the RIA of somatostatin, recovery studies were done on synthetic somatostatin added to either tissue homogenates or extracts using 2 mol/l acetic

*See appendix for details of t test.

Table 11. Comparison of extraction of IRS with 2 mol/l acetic acid and 0.1 mol/l hydrochloric acid

Tissue	2 mol/l acetic acid		0.1 mol/l hydrochloric acid	
	wet weight mg	IRS pg/mg wet wt	wet weight mg	IRS pg/mg wet wt
Hypothalamus	24.05 ± 1.89	1800 ± 190	26.03 ± 1.56	1820 ± 200
Cerebral cortex	41.42 ± 1.98	519 ± 44	38.12 ± 3.45	526 ± 36
Pancreas	326.1 ± 21.5	309 ± 40	341.2 ± 22.7	139 ± 16[a]
Stomach	341.4 ± 48.5	803 ± 105	347.8 ± 39.8	351 ± 46[a]

Data are expressed as mean ± standard error of the mean (SEM), n = 8.

[a] $p<0.01$, two tailed t-test, comparing methods of extraction.

Table 12. Effect of duration of storage on tissue IRS concentration

Tissue	Days of storage at -20°C	Extraction Solvent	
		Acetic Acid (2 mol/l)	Hydrochloric Acid (0.1 mol/l)
Hypothalamus	6	1800 ± 190	1820 ± 200
	460	1330 ± 420	458 ± 240[a]
Cerebral cortex	2	519 ± 44	526 ± 36
	324	398 ± 34[b]	181 ± 24[a]

Data are expressed as the mean ± SEM (n=8)
[a] $p<0.001$, $p<0.05$, two tailed t test, comparing short term storage to long term storage.

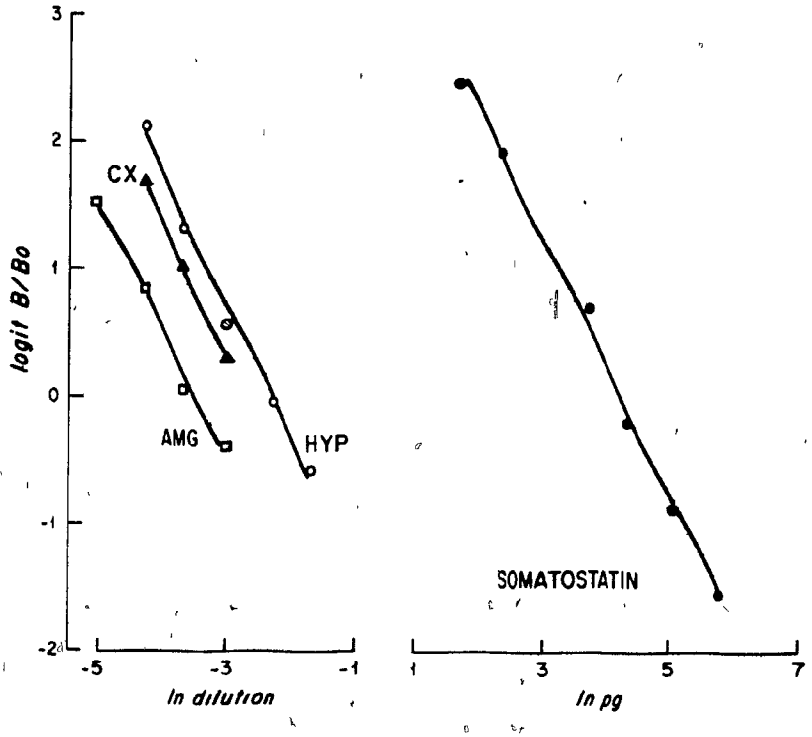


Figure 8. Binding of [^{125}I-Tyr^{1}]somatostatin to antiserum as a function of dilutions of rat brain extracts and pg of synthetic somatostatin. B/Bo is defined in Figure 7. ln, natural logarithm. Each point is the mean of duplicate determinations. The lines were determined by a nonweighted least squares linear regression analysis.

	slope	Est SE of slope	95% conf. slope	y int.	r
somatostatin	-0.977	0.031	±0.086	4.13	0.9981
hypothalamus (HYP)	-0.971	0.059	±0.186	-2.23	0.9968
amygdala (AMG)	-0.946	0.076	±0.325	-3.31	0.9935
cortex (CX)	-0.995	0.017		-2.67	0.9997

Est SE slope, estimated standard error of the slope
95% conf. slope, 95% confidence limits of the slope
y int, y intercept of linear regression line
r, correlation coefficient

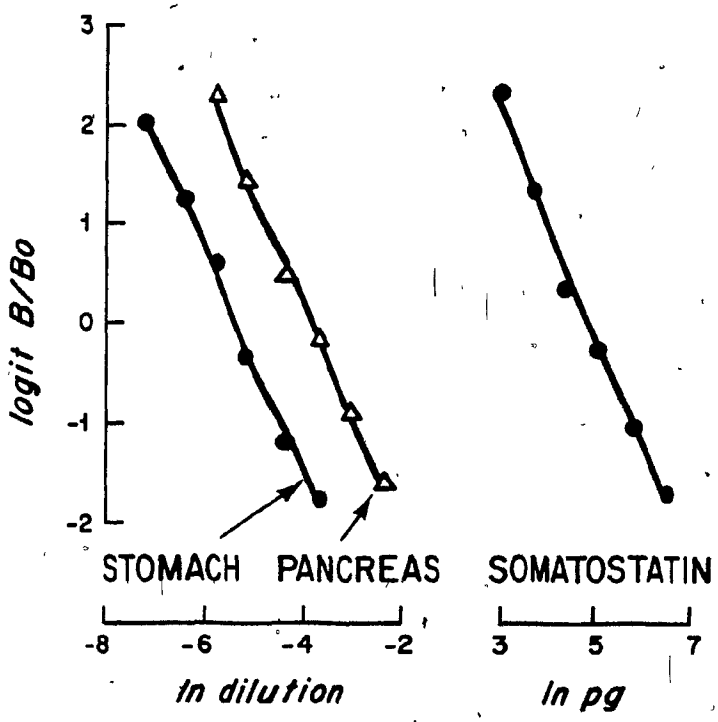


Figure 9. Binding of [^{125}I-Tyr^{1}]somatostatin to antiserum as a function of dilutions of tissue extracts and pg of synthetic somatostatin. B/Bo is defined in Figure 7. The lines were analysed as in Figure 8.

	slope	Est SE of slope	95% Conf. slope	y int.	r
somatostatin	-1.150	0.038	±0.107	5.63	0.9963
pancreas	-1.112	0.034	±0.094	-4.21	0.9982
stomach	-1.122	0.028	±0.079	-5.96	0.9983

acid as extraction solvent (Table 13). In each case the amount of synthetic somatostatin added was approximately the same as the amount of endogenous IRS expected to be present in the aliquot of homogenate or extract. The near complete recoveries for each tissue indicate that somatostatin is not lost or degraded before or during the RIA procedure.

The concentrations of IRS in selected rat brain and gastrointestinal regions are given in Tables 11 and 14. The highest concentrations of IRS were present in the hypothalamus, anterior hypothalamic-preoptic area and gastric antrum and the lowest levels were in the cerebellum and olfactory bulb. IRS was not detectable in liver extracts.

B. BIOASSAY FOR SOMATOSTATIN

1. Materials and Methods

a. Materials. The sources of the materials are as follows: incubation dishes (35x10mm), Corning Glass Works; Spinner Flask, Bellco; DMEM 1885 culture medium, fetal calf serum, penicillin G, and streptomycin, Gibco; collagenase (CLS IV), Worthington; deoxyribonuclease (DNAse), BSA (Cohn fraction V), and HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid), Sigma Chemical Co; RIA kit for rat GH, National Institute for Arthritis, Metabolism and Digestive Diseases, U.S.A.; Normal monkey serum and goat antiserum against monkey gamma globulin, Antibodies Incorporated.

b. Method of bioassay. The bioassay of GH release inhibiting activity essentially uses the dispersed anterior pituitary cell culture method developed by Vale and colleagues (89). Male Charles River CD rats weighing 175-200 g were decapitated, and the anterior pituitary gland was removed intact and placed in 0.025 mol/l HEPES buffer pH 7.2 containing BSA (Cohn fraction V) 0.5% (wt/vol), DNAse 10 µg/ml, NaCl 137mmol/l, KCl 5 mmol/l, Na_2HPO_4 0.7 mmol/l, glucose 11 mmol/l, penicillin 0.3 mg/ml and streptomycin 0.1 mg/ml.

The tissue was minced manually using scapel blades and washed with HEPES buffer to remove residual blood. The pituitary cells were dispersed at 37°C in a spinner flask in the above HEPES buffer containing collagenase 0.4% (wt/vol). Manual trituration was performed with a sterile Pasteur

Table 13. Recovery of synthetic somatostatin added to homogenates or extracts of rat Tissues

Tissue fragment	wet weight mg	ratio $\frac{\text{somatostatin recovered}}{\text{somatostatin added}}$	
		Homogenate	Extract
Hypothalamus	12.62 ± 0.50	1.01 ± 0.11	0.92 ± 0.07
Amygdala	23.60 ± 1.13	0.87 ± 0.09	0.96 ± 0.12
Cerebral cortex	29.96 ± 1.56	1.00 ± 0.07	0.90 ± 0.08
Pancreas	464.5 ± 13.4		0.94 ± 0.06
Stomach (antrum)	196.6 ± 9.0		1.01 ± 0.04
Duodenum	355.2 ± 13.2		0.96 ± 0.07
Jejunum	267.3 ± 8.6		0.95 ± 0.08
Colon	254.1 ± 17.4		1.02 ± 0.07

Data are expressed as Mean ± SEM,

n=10, except hypothalamus extract (n=7), and colon extract (n=8)

In all cases the tissue fragments had been heated to 100°C for five min in 2 mol/l acetic acid prior to homogenization.

Table 14. IRS concentrations in brain and gastrointestinal tissues of the rat

	Fragment wet wt mg	IRS ng/mg wet wt	IRS ng/mg protein
Anterior hypothalamic-preoptic area	17.79 ± 0.68	1.32 ± 0.04	24.7 ± 1.0
Amygdala	56.21 ± 2.06	0.978 ± 0.028	19.6 ± 0.8
Cerebral cortex	114.38 ± 2.16	0.491 ± 0.039	10.3 ± 0.3
Olfactory bulb	58.51 ± 1.62	0.071 ± 0.006	1.12 ± 0.17
Brainstem	183.6 ± 5.37	0.311 ± 0.013	9.26 ± 0.48
Cervical spinal cord	137.0 ± 3.96	0.401 ± 0.013	13.2 ± 0.4
Cerebellum	261.1 ± 4.10	0.047 ± 0.003	1.11 ± 0.09
Pancreas	362.4 ± 18.0	0.332 ± 0.036	
Stomach (antrum)	331.9 ± 13.7	0.869 ± 0.058	
Duodenum (proximal)	376.1 ± 17.9	0.096 ± 0.013	
Jejunum	284.7 ± 9.6	0.096 ± 0.17	
Colon	294.8 ± 8.2	0.130 ± 0.022	
Liver	155.1 ± 8.9	<0.01	

Data are expressed as mean ± SEM.
n=8 except for liver where n=2.
The concentration of IRS is not corrected for recovery.
The extraction solvent was 2 mol/l acetic acid and the tissue fragments were heated to 100°C for five minutes before homogenization.

pipette that had had its opening reduced to <1 mm by a Bunsen burner. The dispersion process yielded a opalescent suspension after approximately 1 h. The cells were next centrifuged for 5-10 min at 800 × *g* and washed once with incubation medium consisting of DMEM 1885, with added fetal calf serum 10% (vol/vol), glucose 11 mmol/l, HEPES 15 mmol/l, penicillin G 0.3 mg/ml, and streptomycin 0.1 mg/ml. The cells were portioned out in 2 ml fractions containing approximately 2 x 10^5 cells to 35 x 10 mm culture dishes. After preincubation for 3 days at 37°C in 95% air-5% CO_2 and 100% humidity the medium was removed and the cells, which had attached to the bottom of the dish, were washed once with incubation medium composed as above except that the fetal calf serum was replaced with BSA (RIA grade) 0.1% (wt/vol) to avoid the presence of plasma peptidases that could degrade somatostatin.

The actual bioassay consisted of adding 800 µl of fresh culture medium, BSA 0.1% (wt/vol) per dish followed by 200 µl of either: (1) a standard solution of 20-1280 pg synthetic somatostatin diluted in incubation medium, BSA (RIA grade) 0.1% (wt/vol) or, (ii) neutralized experimental samples diluted similarly as synthetic somatostatin. After incubation for 3 h, the culture medium was aspirated and stored at -20°C for subsequent RIA for rat GH.

c. RIA of rat GH. RIA of rat GH was carried out using the materials and instructions included in the kit provided by the National Institutes of Arthritis, Metabolic and Digestive Diseases, USA.

The standard rat GH preparation was GH-RP-1. The standard dose response curve ranged from 0.156 to 20 ng rat GH per tube. The between-assay coefficient of variation was 11.3% for a sample containing 1.87 ng of rat GH (n=20). Separation of bound and free ^{125}I-labelled rat GH was achieved by a double antibody technique using normal monkey serum and goat antiserum raised against monkey gamma globulin. The incubation media from the bioassays were diluted in rat GH RIA buffer and assayed in duplicate.

2. Results

A standard dose response curve of the inhibition of rat GH release from anterior pituitary cells by increasing concentrations of synthetic somatostatin is shown in Figure 10. A 50% maximum inhibition of rat GH

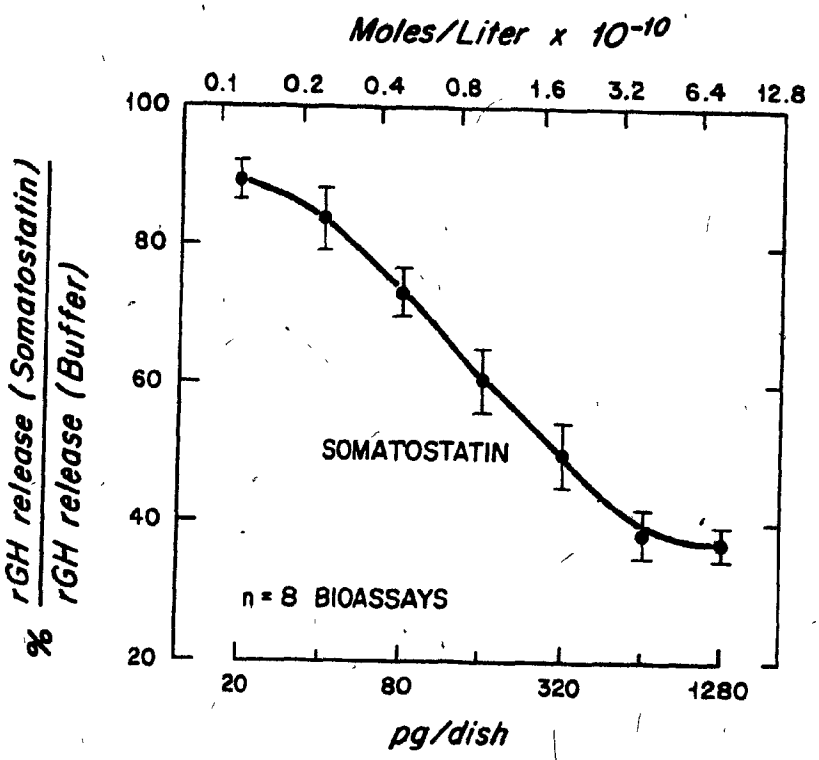


Figure 10. Release of GH from cultured rat anterior pituitary cells as a function of somatostatin concentration in the incubation medium. The vertical axis shows the ratio of GH released in the presence of somatostatin to that released in the absence of somatostatin (buffer alone) expressed as a percentage. The incubation conditions are given in materials and methods. Each point is the mean of eight bioassays and the vertical lines indicate the SEM.

release occurred at a somatostatin concentration of 6.5×10^{-11} mol/l. The maximum inhibition of rat GH release achieved was to 38% of basal release.

C. AFFINITY CHROMATOGRAPHY OF SOMATOSTATIN

1. Materials and Methods

The sources of the following are: cyanogen bromide-activated Sepharose 4B, Pharmacia Fine Chemicals; porcine SME fragments, Oscar Mayer Co.

An immunoglobulin fraction of the SS-AS was prepared by dropwise addition of a saturated solution of $(NH_4)_2SO_4$, adjusted to pH 6.8 with NaOH, to the antiserum at 4°C to yield a final concentration of 40% (vol/vol) saturated $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 12,000 × *g* at 4°C for 20 min, dialyzed for 2 days against a thrice replenished 0.01 mol/l sodium phosphate buffer (pH 7.4), 0.15 mol/l NaCl, and stored at -20°C in the above buffer.

A volume of 0.5 ml SS-AS or nonimmune serum or, alternatively, the $(NH_4)_2SO_4$-precipitated immunoglobulins prepared from these sera were incubated with 4g of cyanogen bromide-activated Sepharose 4B for 2 h at room temperature (21°C) in a 0.1 mol/l $NaHCO_3$ buffer (pH 8.0), 0.5 mol/l NaCl with continuous end-over-end mixing. After the gel was washed on a Buchner funnel with the $NaHCO_3$ buffer, it was incubated with 1 mol/l ethanolamine dissolved in the $NaHCO_3$ buffer, pH 8.0, similarly as above. The reacted gel was washed with three cycles of 0.1 mol/l sodium acetate buffer (pH 4.0), 1.0 mol/l NaCl followed by 0.1 mol/l sodium borate buffer (pH 8.0), 1.0 mol/l NaCl. Finally, the gel was washed with 2 mol/l acetic acid or 0.1 mol/l HCl to remove any somatostatin bound to the coupled immunoglobulin, rewashed with 0.1 mol/l sodium phosphate buffer (pH 7.4), and stored in the sodium phosphate buffer containing 0.01% sodium azide at 4°C.

Affinity chromatography was performed at 4°C by first washing a column of the prepared gel with 0.1 or 0.01 mol/l sodium phosphate buffer, pH 7.4, then applying an extract which had been neutralized to pH 7.4 with NaOH. Following the extract, the column was rewashed with the sodium phosphate buffer. The bound IRS was eluted with either 2 mol/l acetic acid or 0.1 mol/l HCl. After rewashing with buffer, columns have been reused up to

three times.

2. Results

During preliminary experiments it became evident that when SS-AS was coupled to Sepharose it carried along a small quantity of somatostatin noncovalently attached to itself. This somatostatin could be eluted from the affinity columns by acid (Figure 11, Blank 1). It is important to note that the affinity gel used in Figure 11 had not been previously washed with 0.1 mol/l HCl or 2 mol/l acetic adjc during its preparation. Washing the column a second time with neutral buffer and acid (Figure 11, Blank 2) showed that virtually all the somatostatin had been removed by the previous acid wash. For all subsequent affinity experiments an acid wash was included in the preparative steps as described in materials and methods.

The third elution profile of the same column in Figure 11 shows the recovery of IRS from rat plasma by affinity chromatography. Previous studies by Drs. Brazeau and Epelbaum had demonstrated that incubation of [^{125}I-Tyr^{1}]somatostatin with unextracted rat plasma resulted in inhibition of binding of [^{125}I-Tyr^{1}]somatostatin to antiserum, but that acetic acid extraction of rat plasma prevented the inhibition of binding (455). Therefore, for the experiment shown in Figure 11, 9 ml of pooled hepatic venous plasma was obtained from pentobarbital-anesthetized rats and extracted with an equal volume of 2 mol/l acetic acid. After centrifugation of the extract at 2000 × g for 30 min at 4°C the supernatant was neutralized with NaOH to pH 7.4 and applied to the immunoaffinity column. With acid application to the column a peak of IRS eluted. The recovered IRS corresponded to a concentration of 360 pg IRS/ml plasma.

To assess whether sufficient SS-AS to interfere with the RIA became uncoupled from the affinity gel under chromatographic conditions, 6 ml 0.01 mol/l sodium phosphate buffer, pH 7.4, were washed over 1.75 h through a column of 1 ml gel which had been coupled with 0.067 ml SS-AS. The eluate was lyophilized, resuspended in 1.5 ml distilled H_2O, and tested for its ability to bind [^{125}I-Tyr^{1}]somatostatin under the conditions of the RIA. No binding activity was detected in the redissolved eluate. The binding capacity of the affinity gel was estimated by eluting a solution of synthetic somatostatin (100 ng/ml) dissolved in 0.01 mol/l sodium phosphate buffer,

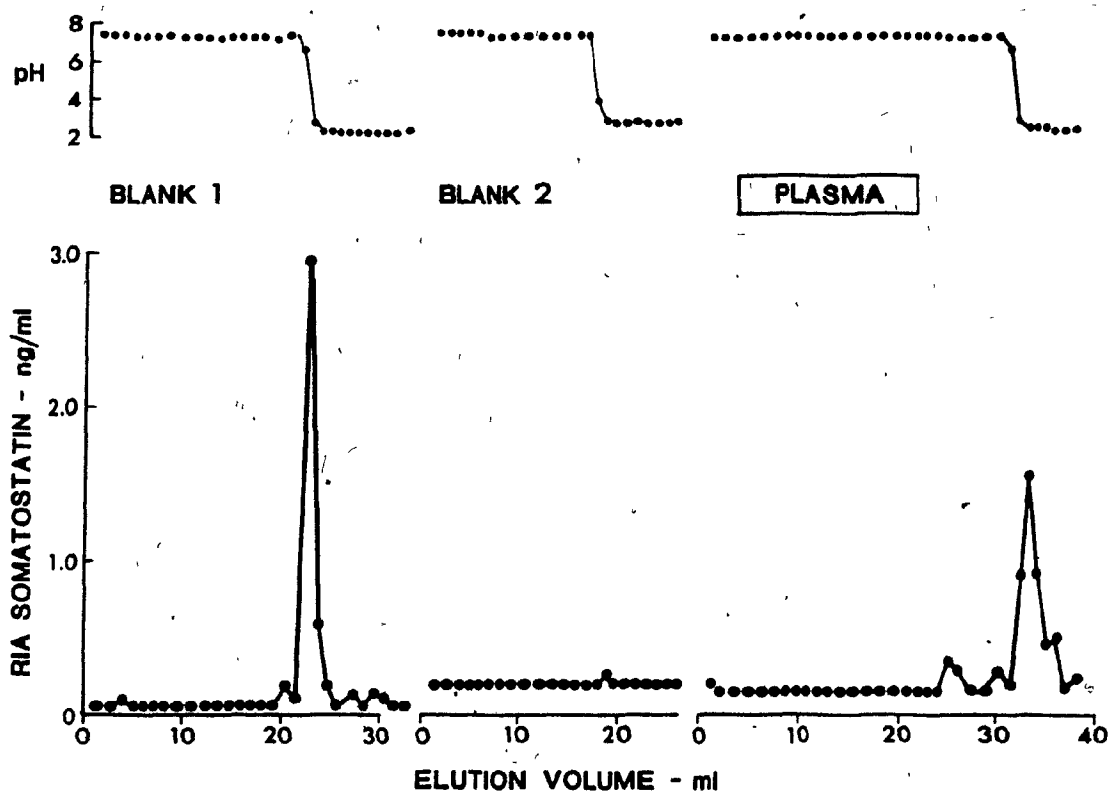


Figure 11. Affinity chromatography experiments done as described in materials and methods except that the affinity gel had not previously received an acid wash. A 6 × 0.7 cm column was used with a flow rate of 7 ml/hr. The pH and concentrations of radioimmunoassayable somatostatin (ng/ml) are plotted against the elution volume of three experiments. The position of acid elution in each profile is indicated by the drop of pH. Blank 1: No experimental extract was applied. On elution with 2 mol/l acetic acid a peak of IRS is evident. Blank 2: A second acetic acid wash shows that virtually all the IRS had been removed. Plasma: Application of a neutralized extract of rat plasma.

pH 7.4, through a column of gel and measuring the amount of IRS in the eluate fractions. Somatostatin was completely adsorbed to a column originally coupled with 0.1 ml SS-AS until a total of 1.2 µg of somatostatin were applied. Application of larger amounts of somatostatin to the column resulted in leakage of somatostatin into the eluate. The recovery of IRS after acid elution was 82.4 ± 2.5%, (mean ± SEM) of synthetic somatostatin (10 ng) applied to six affinity columns each containing 300 µl gel.

The applicability of affinity chromatography for the separation of IRS from brain tissue extracts was assessed using a 0.2 mol/l acetic acid extract of 10 porcine SME fragments (Figure 12). The applied IRS which bound to the affinity column was eluted after acid addition with a recovery of 87%. A control chromatography using nonimmune sheep serum coupled to Sepharose resulted in the elution of 5% of the applied IRS after the addition of acid.

Subtraction of IRS from extracts of porcine SME by affinity chromatography also reduced the GH release-inhibiting activity of the residual extract. An extract of porcine SME was chromatographed using columns of immobilized SS-AS and nonimmune sheep serum, as shown in Figure 12. The first large protein peak eluted from the control nonimmune serum column contained 49.8 ng IRS/SME equivalent. This material gave a dose-related inhibition of GH release (Table 15, group IV) comparable to that of synthetic somatostatin (group II). The material in the large protein peak eluted from the SS-AS-coupled column contained no detectable IRS and did not cause a dose-related inhibition of GH release (group III). Although this residual IRS-free extract produced a small mean decrease of GH release compared to the buffer control (group I), only one point (1.6×10^{-2} SME fragments) reached statistical significance ($p<0.05$, by two-tailed t test). Higher concentrations of the IRS-free extract did not significantly inhibit the release of GH.

D. RETINAL SOMATOSTATIN-LIKE MATERIAL

1. Materials and Methods

a. Collection and extraction of rat retinas. Male Charles River CD

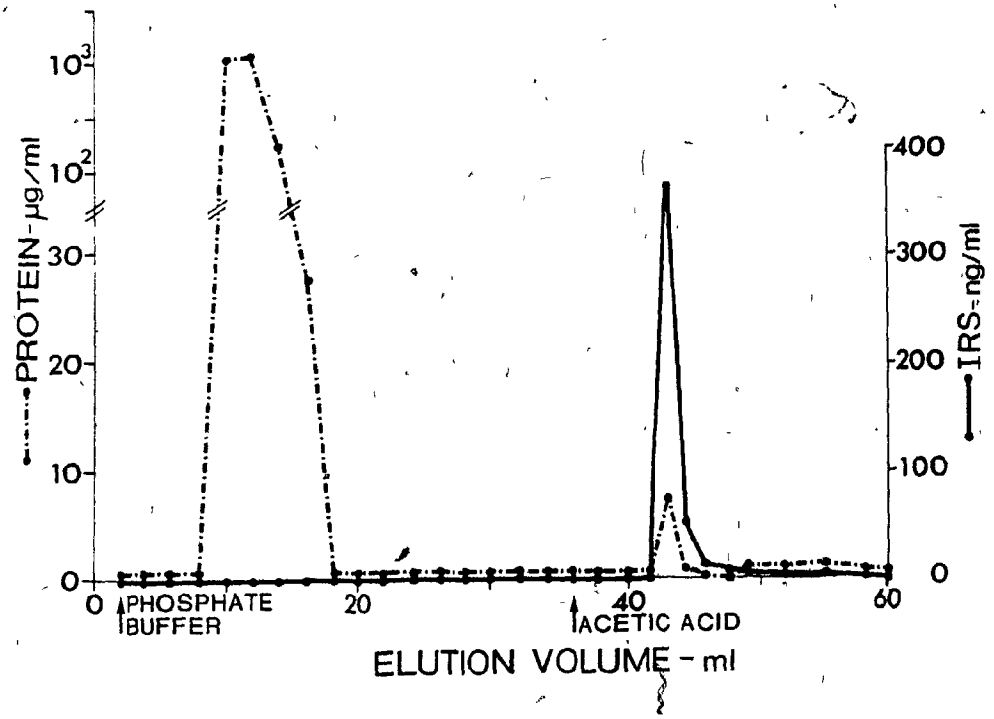


Figure 12. Affinity chromatography of a 0.2 mol/l acetic acid extract of 10 porcine SME fragments done as described in materials and methods. The column dimensions were 6 × 0.7 cm and the flow rate was 9 ml/hr. The large protein peak corresponds to the position of application of the extract. The volumes of elution with sodium phosphate 0.01 mol/l buffer, pH 7.4, and 2 mol/l acetic acid are indicated.

Table 15.

Effect on GH release of an extract of porcine SME from which IRS had been subtracted by immunoaffinity chromatography

Groups	SME fragment equivalents/dish	IRS in extract (ng/dish)	rGH released (ng/ml)
I. Buffer control (no somatostatin)			962 ± 18
II. Synthetic somatostatin (ng/dish)			
0.03			883 ± 20[a]
0.1			868 ± 39
0.3			805 ± 25[b]
1.0			671 ± 34[b]
3.0			373 ± 5[b]
III. Extract eluted through immobilized antiserum			
	6.4×10^{-3}	ND[c]	1000 ± 104
	1.6×10^{-2}	ND	868 ± 15[a]
	4.0×10^{-2}	ND	867 ± 65
	1.0×10^{-1}	ND	880 ± 42
IV. Extract eluted through immobilized nonimmune serum			
	6.4×10^{-3}	0.3	933 ± 134
	1.6×10^{-2}	0.8	730 ± 28[b]
	4.0×10^{-2}	2.0	568 ± 66[b]
	1.0×10^{-1}	5.0	292 ± 25[b]

Data are expressed as the mean ± SE (n = 5 incubation dishes for buffer control and synthetic somatostatin samples; n = 3 for porcine SME extracts).

[a] $P < 0.05$ *vs.* buffer control (by two-tailed *t* test).

[b] $P < 0.01$ *vs.* buffer control (by two-tailed *t* test).

[c] Nondetectable.

rats were maintained as described above with lights on from 0600 to 1800 h. Rats that were used for the quantitative determinations of IRS content per individual retinal fragment ranged in weight from 200 to 250 g and were killed by decapitation or ether inhalation between 0930 and 1130 h. The pools of retinal tissue used for the chromatographic studies of IRS were obtained predominantly from rats weighing 200–250 g, but also included retinas from rats weighing up to 600 g, and were collected at various times between 0830 and 1600 h. The eyes were removed after death, the intact retinas were dissected free under visualization with a dissecting microscope, and each retinal fragment was placed in 0.5 ml of 0.1 mol/l HCl. Dissection of the intact retina resulted in a tissue fragment that included a superficial portion of the ciliary body adherent to the retina. Microscopic study of hematoxylin/eosin-stained cross sections of the retinal tissues indicated that the plane of dissection extended between the photoreceptor cell and the choroid layers. This confirmed that all cellular layers of the retina were included in the tissue fragment. The fragments were homogenized, centrifuged and stored as described above. The protein content of retinal extracts was determined by the fluorometric method as described above.

Hydrochloric acid was used as the extraction solvent for the retinal experiments in order to avoid the relatively large amount of salt which forms on neutralization of the more concentrated acetic acid extractant. Studies using particular rat retinal extracts were completed within four weeks to minimize the reduction of IRS concentration that occurs with prolonged storage in HCl.

The recovery of synthetic somatostatin added to eight separate retinal extracts was 105 $\pm$ 10% (mean $\pm$ SEM), indicating that no loss of immunological activity of somatostatin occurred before or during the RIA.

b. Gel filtration chromatography. Sephadex G-25 fine (Pharmacia Fine Chemicals) was swollen overnight in 0.2 mol/l acetic acid containing 0.1% HSA (Cohn fraction V). Chromatography was performed on a 1.1 × 50 cm column at 4°C with an eluent of 0.2 mol/l acetic acid, 0.1% HSA (Cohn fraction V), a flow rate of 10 ml/h, and a fraction size of 1.0 ml. A portion of each fraction was neutralized with NaOH and assayed for IRS. Synthetic

somatostatin (200 ng) was chromatographed under similar conditions with a recovery of 51%. The incomplete recovery is probably due to binding of the peptide to the gel or to loss of immunological activity.

c. Experimental models of retinal degeneration. In order to obtain clues to the cellular localization of IRS in the rat retina we, in collaboration with Dr. M. Brownstein, Laboratory of Clinical Science, National Institutes of Mental Health, U.S.A., studied the retinal concentration in rats with experimental retinal degeneration. IRS was measured in adult (250 *g*) PETH (pink-eyed, tan-hooded) rats and in albino (Osborne-Mendel) controls. PETH rats have inherited retinal dystrophy which begins to manifest itself in the third postnatal week (456,457). The adult animals have few if any rods and gradually lose most of their cones as well. Thus, if IRS were confined to photoreceptor elements in the retina, PETH rat retinas should have little of this material.

In addition to studying PETH rats, we also examined the retinas of rats that had been subjected to intracranial bilateral optic nerve transections. Rats (250 *g*) were placed in a Kopf stereotaxic device (5°, nose down) and a 3-mm knife with its blade in the coronal plane was centered over the midline and inserted into the brain 10-11 mm rostral to the interauricular line. The knife was lowered until it reached the base of the brain and pressed into the bone so that it cut both optic nerves. The animals were killed one year after the operation. After this time all of the ganglion cells had degenerated (458) and the optic nerves and tracts had completely atrophied. The retinas of the surgically prepared rats would be expected to have no IRS if it were localized exclusively in the ganglion cells.

d. Collection and extraction of human retinas. Human retinas were obtained by Dr. M. Senterman, Dept. of Pathology, Montreal General Hospital and Drs. K. Hoyte and A. Hakim, Dept. of Neuropathology, Montreal Neurological Institute. Pertinent descriptive data on the patients is included in Tables 17 and 19. The posterior globe was exposed by an intracranial approach during autopsy and a portion of the retina was dissected free of the uvea and sclera. The retina was placed in 1-2 ml of 0.1 mol/l HCl and stored frozen before extraction as described for the rat retina. In the case of

the retinal extracts obtained from patients listed in Table 17, the protein concentration was determined according to the method of Lowry *et al* (459) because the fluorometric method was not available at that time. BSA (RIA grade) was the reference standard as before. The hypothalami were dissected from patients 13 and 14 (Table 19) by Drs. K. Hoyte and A. Hakim, Dept. of Neuropathology, Montreal Neurological Institute, and were extracted as for the retinas in 0.1 mol/l HCl (tissue to acid ratio<0.1 wt/vol) with heating to 100°C for 5 min.

2. Results

a. Concentration of IRS in rat retina. The concentrations of IRS were 612 ± 43 (mean ± SEM) pg per retina or 0.621 ± 0.044 pg/µg of protein for a total of 16 retinas obtained from 10 rats killed by decapitation and 601 ± 22 pg per retina or 0.577 ± 0.022 pg/µg of protein for 19 retinas obtained from 10 rats killed by ether inhalation. The differences between the two groups were not statistically significant ($p<0.1$, two-tailed t test).

b. Partial characterization of rat retinal somatostatin-like activity.

Figure 13 represents the affinity chromatography of a pool of retinal extracts containing a total of 37.9 ng of IRS. The concentration of IRS in the elution fractions corresponding to the application of the sample was below the RIA limit of detection (200 pg/ml). However, 52% of the applied IRS eluted when the column was washed with HCl. The affinity-purified retinal IRS produced a displacement curve of [^{125}I-Tyr^{1}]somatostatin from SS-AS that was similar to those of synthetic somatostatin and rat hypothalamus extracted in HCl by the same method as used for retinas (Figure 14). The displacement lines of crude retinal extracts were also parallel to that of synthetic somatostatin (data not shown).

Figure 15 indicates the dose-response curves in the somatostatin bioassay as determined by inhibition of rat GH released into the incubation medium compared to varying quantities of somatostatin, crude retinal extract, and the affinity-purified IRS obtained from the chromatography described in Figure 13. All three samples produced a dose-related inhibition of release of rat GH. The ratios of somatostatin-like activity

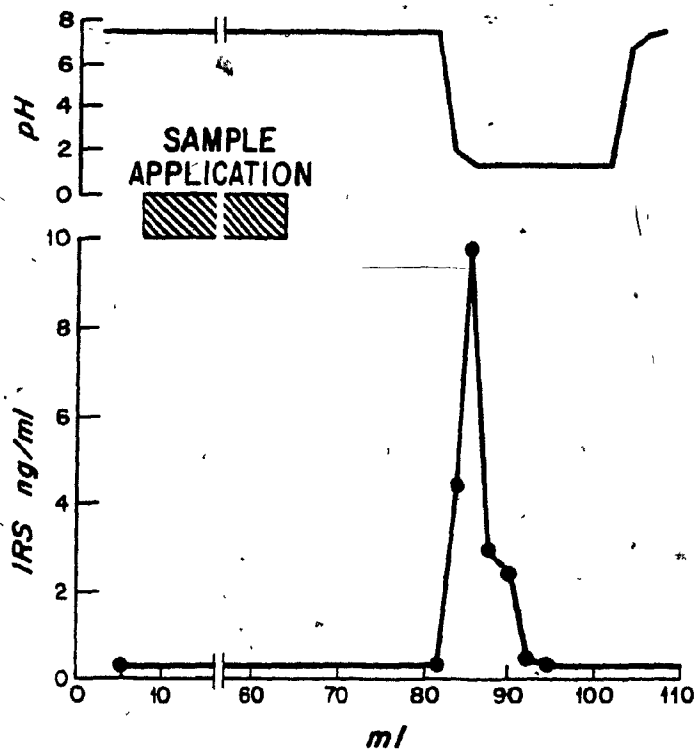


Figure 13. Affinity chromatography of rat retinal IRS. The pH and concentration of IRS (ng/ml) in each 2 ml elution fraction are plotted against the cumulative elution volume. A total of 37.9 ng of IRS was applied in a volume of 55 ml to a 6.4 × 0.7 cm column run at 12.5 ml/hr.

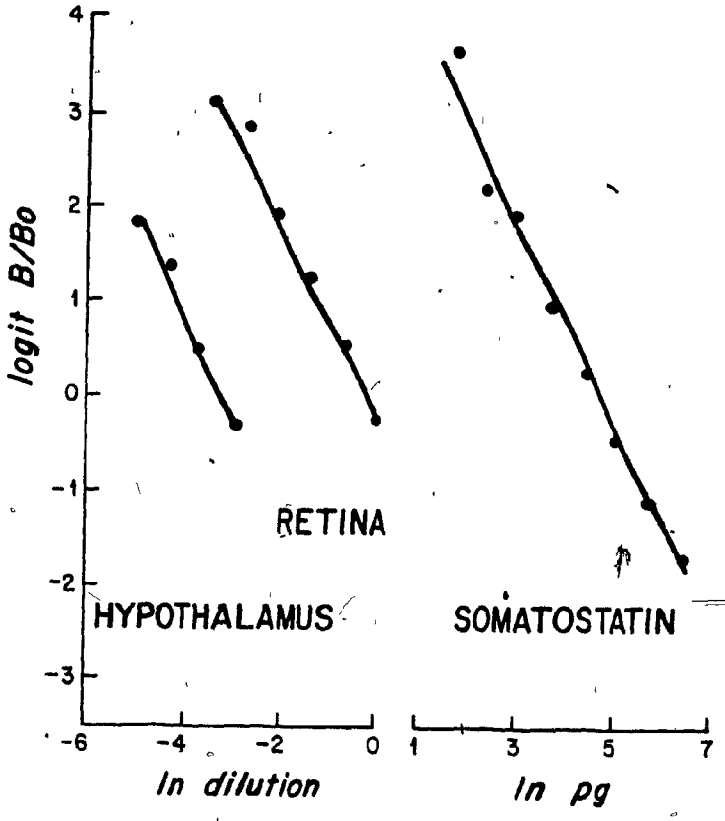


Figure 14. Binding of [^{125}I-Tyr^{1}]somatostatin to antiserum as a function of dilutions of rat hypothalamic extract, affinity-concentrated rat retinal IRS, and pg of synthetic somatostatin. B/Bo is defined in Figure 7. The lines were analysed as in Figure 8.

	slope	Est SE of slope	95% Conf. slope	y Int.	r
somatostatin	-1.063	0.053	±0.129	4.99	0.9927
rat retina	-1.019	0.064	±0.170	-0.22	0.9927
rat hypothalamus	-1.101	0.102	±0.435	-3.64	0.9927

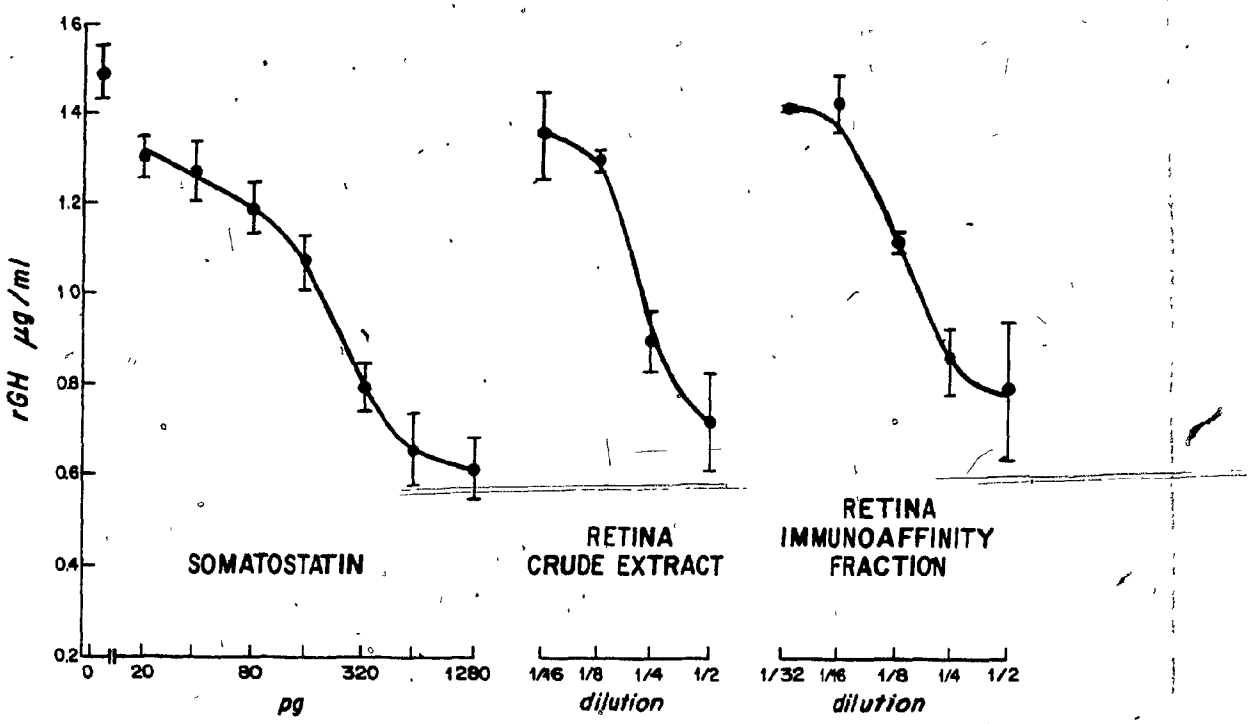


Figure 15. Curves relating the concentration of rat GH (rGH) released from cultured rat anterior pituitary cells into their culture media and pg of synthetic somatostatin or dilutions of a crude retinal extract and affinity-purified IRS added to the incubation media. Each point represents the mean of rat GH release in six incubation dishes for the synthetic somatostatin curve and the buffer control (0) and three dishes for the retinal samples. The vertical lines represent the SEM.

determined by bioassay to that determined by RIA were 0.84 and 0.79 for two separate affinity experiments and bioassays. The bioassay/RIA ratios of somatostatin-like activity assayed in two separate crude retinal extracts were 1.53 and 2.09.

Figure 16 shows the chromatographic behavior on gel filtration of the IRS purified by affinity chromatography. A total of 45% of the IRS that eluted from the affinity column was recovered after lyophilization and gel filtration chromatography. Approximately 96% of the recovered IRS eluted in a peak at a volume coinciding with that of synthetic somatostatin.

c. Effect of retinal degenerations on IRS concentration. As shown in Table 16, the concentration of retinal IRS, expressed as pg per µg of protein, was significantly increased in PETH rats with hereditary degeneration of photoreceptor cells and in rats studied one year after transection of the optic nerves. A decrease in the retinal protein concentration of the experimental groups accounted for the increase in retinal IRS concentration.

d. IRS in the human retina. The concentrations of IRS in retinal extracts from 11 subjects are given in Table 17. All extracts were studied in the same RIA. The concentration of IRS was compared by linear regression analysis to the age of the patient at death, the time interval between death and the collection of the retina, and the time interval between the collection of the retina and RIA for somatostatin (Figure 17). The significance of the difference between the slopes of the regression lines and zero was determined by a two-tailed t test using the estimated standard error of the slopes (454). The slope of the line relating retinal IRS concentration and the time interval between collection of the retina and RIA was significantly different from zero ($p<.01$) indicating an inverse relationship between retinal IRS concentration and time of storage in 0.1 mol/l HCl. No statistically significant relationship could be demonstrated between retinal IRS concentration and age at death or the time interval between death and collection of the tissue (Table 18).

Crude extracts of human retinas and hypothalami produced RIA displacement lines that were parallel to that of synthetic somatostatin (Figure 18). Portions of the retinal extracts from the 11 patients described in Table 17 were pooled, lyophilized, and resuspended in 0.1 mol/l sodium

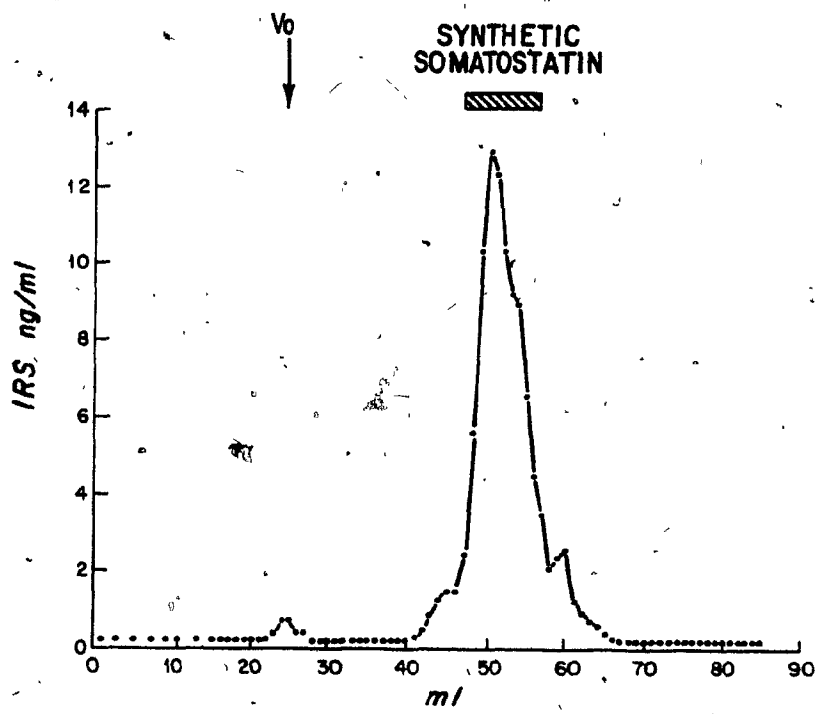


Figure 16. Gel filtration chromatography of affinity-purified retinal IRS. The concentration of IRS per ml of elution fraction is compared to the cumulative elution volume. Vo is the column void volume. The elution volume of synthetic somatostatin is indicated by a crosshatched bar. Experimental conditions are described in materials and methods.

Table 16. Effect of retinal degeneration on IRS concentration

Rats (n = 6)	Protein µg/extract	IRS pg/extract	IRS pg/µg protein
Control	921 ± 20	879 ± 80	0.96 ± 0.09
Hereditary photoreceptor degeneration	454 ± 28[a]	725 ± 39	1.46 ± 0.16[b]
Optic nerve transection	474 ± 26[a]	1158 ± 168	2.40 ± 0.27[a]

Data are expressed as mean ± SEM.

[a] $p<0.001$.

[b] $p<0.01$, compared to control (two-tailed t test)

Table 17. IRS Concentration in Human Retina

Patient	Retinal IRS (pg/mg protein)	Sex	Age at Death	Time from death to collection of retina (h)	Time from collection of retina to RIA of extract (days)	Cause of Death
1	763	M	75	7.5	118	Bronchopneumonia
2	492	M	74	4.25	118	Chronic Myelocytic Leukemia
3	537	M	71	17	111	Myocardial Infarction
4	600	M	64	28.25	111	Myocardial Infarction
5	572	M	93	13.25	101	Bronchopneumonia
6	727	M	84	3.5	89	Bronchopneumonia
7	1090	F	59	11	77	Myocardial Infarction
8	531	M	70	4.67	77	Myocardial Infarction
9	1480	M	54	10.83	50	Myocardial Infarction
10	1200	F	64	8.5	49	Acute Lymphoblastic Leukemia
11	966	M	26	10.75	43	Malignant Melanoma
Mean			66.7	10.9	85.8	
SEM			5.3	2.1	8.6	

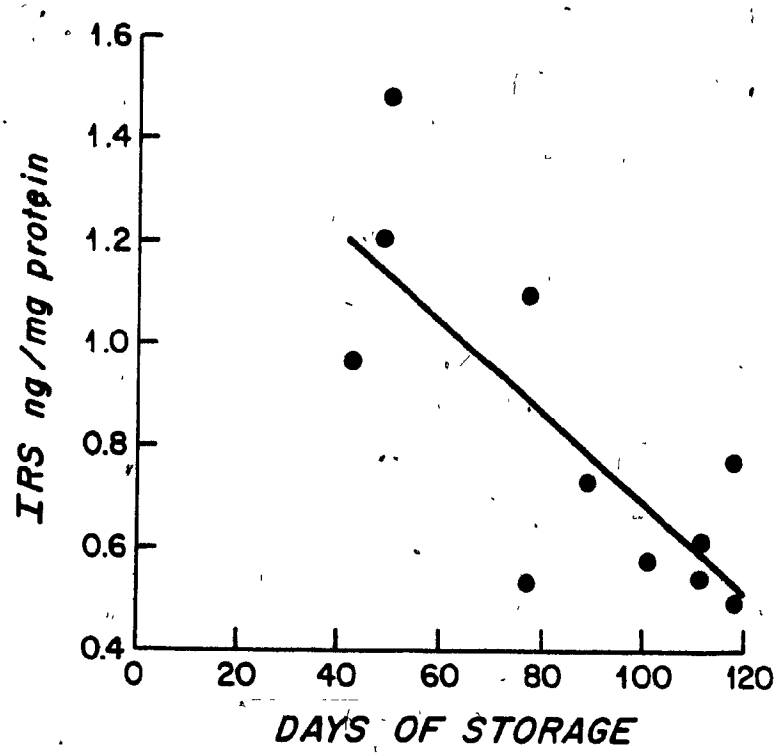


Figure 17. Concentration of IRS in human retinal extracts as a function of days of storage of the retina in 0.1 mol/l HCl at -30°C. Each point represents a retina from a different individual. The line is determined by least squares linear regression analysis.

Table 18. Analysis of the Relationship Between Human Retinal IRS Concentration and Characteristics of the Retinas Shown in Table 17.

Retinal IRS (pg/mg protein) compared to:	Linear Regression Analysis					
	slope	y intercept	correlation coefficient	Est (SE) slope[a]	t_{n-2}[b]	p
Age of patient at death (years)	-9.88	1474	-0.527	5.684	1.738	>0.05
Time interval between death and collection of retina (h)	-4.44	862	-0.096	-	-	-
Time interval between collection of retina and RIA (days)	-8.87	1575	-0.779	2.400	3.693	<0.01

[a] Estimated standard error of the slope.

[b] Two-tailed t test with n-2 degrees of freedom for the difference of the slope from zero.

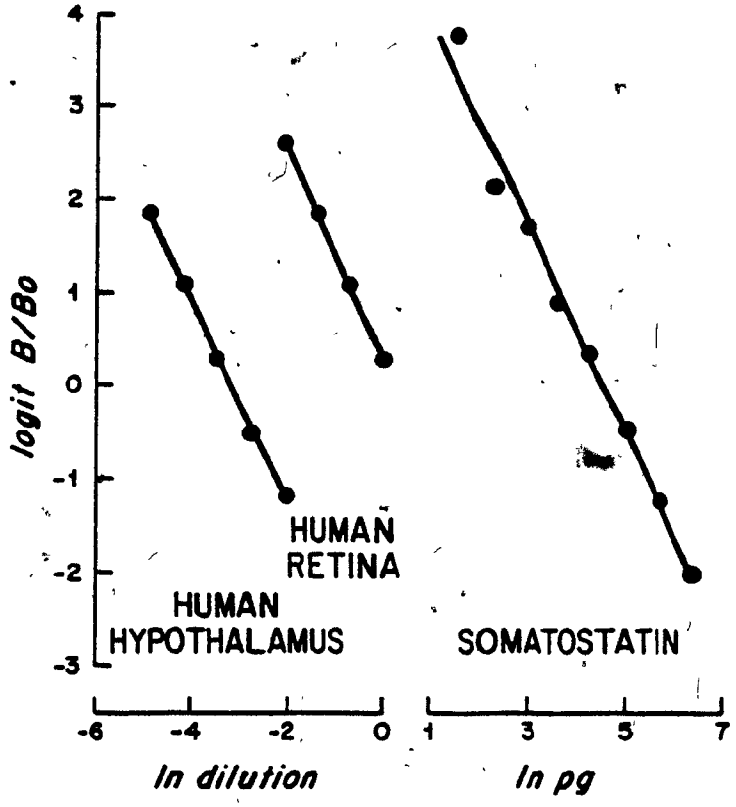


Figure 18. Binding of [^{125}I-Tyr^{1}]somatostatin to antiserum as a function of dilutions of human retina and hypothalamus and pg of synthetic somatostatin. B/Bo is defined in Figure 7. The lines were analysed as in Figure 8.

	Slope	Est SE of slope	95% conf. slope	y int.	r
somatostatin	-1.106	0.059	±0.143	5.11	0.9919
human retina	-1.126	0.037	±0.160	0.29	0.9999
human hypothalamus	-1.114	0.017	±0.055	-3.54	0.9969

phosphate buffer, pH 7.4. The pH was checked to be 7.4 by a pH meter. The suspension was centrifuged at 2000 × *g* for 10 min at 4°C to remove retinal proteins that precipitated at neutral pH. The supernatant, containing 29.5 ng IRS, was applied to an affinity column resulting in a recovery of 61% of applied IRS after elution with 0.1 mol/l HCl (Figure 19). No IRS was detected in the eluate fractions before application of acid.

e. Bioassay of human retinal somatostatin. Extracts of retinas obtained from the patients listed in Table 19 were pooled, lyophilized, resuspended in 0.01 mol/l sodium phosphate buffer (pH 7.4), 0.1 mol/l NaCl and 0.1% (wt/vol) HSA (Cohn fraction V) and neutralized with NaOH using phenol red as a pH indicator. The neutral suspension was centrifuged at 2000 × *g* for 10 min at 4°C to remove a small amount of precipitate. This extract inhibited rat GH release in the bioassay in a dose related manner similarly as did synthetic somatostatin and a HCl extract of human hypothalami (Figure 20). The ratio of somatostatin-like activity determined by bioassay to that determined by RIA of the pooled human retinal extract was 0.88.

E. COMPARISON OF CHROMATOGRAPHIC AND BIOLOGICAL PROPERTIES OF IRS IN HYPOTHALAMIC AND EXTRAHYPOTHALAMIC BRAIN REGIONS

Methods and Results

Fragments of the ME, anterior hypothalamic-preoptic area, amygdala and parietal cortex were obtained from 50 male Charles River CD rats similarly as described above. They were extracted in 2 mol/l acetic acid without heating. Pools of the regional extracts were neutralized with NoOH and subjected to affinity chromatography (Figure 21). The IRS from all four tissue regions adsorbed completely to the affinity columns and was eluted by 2 mol/l acetic acid. The peak of IRS from each region was lyophilized, redissolved in 0.5 ml 0.2 mol/l acetic acid containing 0.1% (wt/vol) HSA (Cohn fraction V), and chromatographed on a Sephadex G-25 column (Figure 22). The affinity-purified IRS from each brain region eluted in four peaks, with the ratio of elution volume to void volume ranging from: peak I, 1.0 - 1.2; peak II, 1.3 - 1.5; peak III, 1.9 - 2.1; and peak IV, 2.2 - 2.4. The largest peak (III) eluted in a position similar

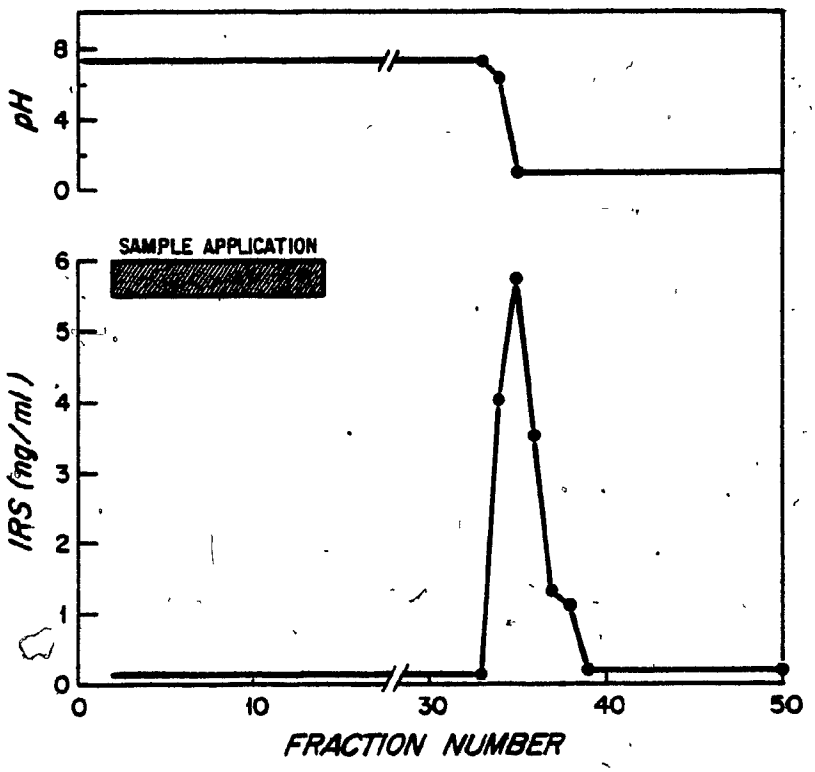


Figure 19. Affinity chromatography of human retinal IRS. A total of 29.2 ng of IRS was applied to a 10 × 0.7 cm column run at 10 ml/hr. The fraction size was 1.06 ml.

Table 19. Details of Patients Providing Retinas for Bioassay of Somatostatin

Patient	Sex	Age at death	Time from death to collection of retinas (hrs)	Cause of death
12	M	68	18.75	Myocardial Infarction
13	F	67	8.0	Chronic Myelocytic Leukemia
14	M	62	24.0	Myocardial Infarction
15	M	72	61.4	Cardiac Arrhythmia
16	M	71	12.0	Carcinoma Lung

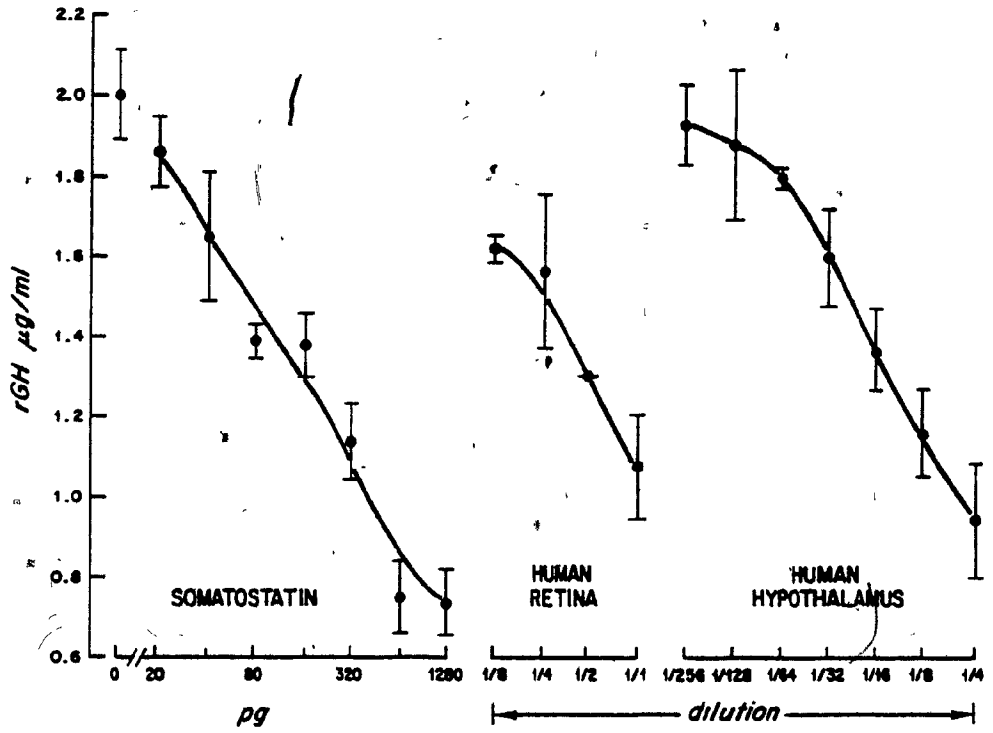


Figure 20. Curves relating the concentration of rat GH (rGH) released from cultured rat anterior pituitary cells into their culture media and pg of synthetic somatostatin or dilutions of human retinal and hypothalamic extracts. Each point represents the mean of rat GH release in four incubation dishes for the synthetic somatostatin curve and the buffer control (0) and three dishes for the tissue extracts. The vertical lines indicate the SEM.

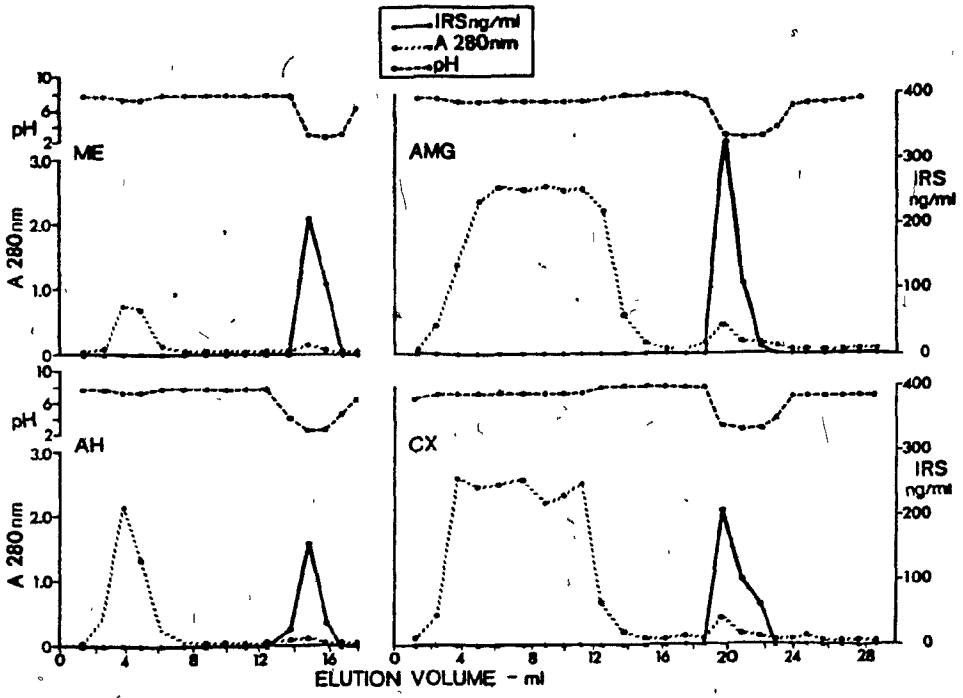


Figure 21. Affinity chromatography of 2 mol/l acetic acid extracts of rat brain regions done as described in materials and methods. The column size was 15 × 0.7 cm. The drop in pH corresponds to the elution with 2 mol/l acetic acid. ME (median eminence), AH (anterior hypothalamic-preoptic area), AMG (amygdala), CX (parietal cortex).

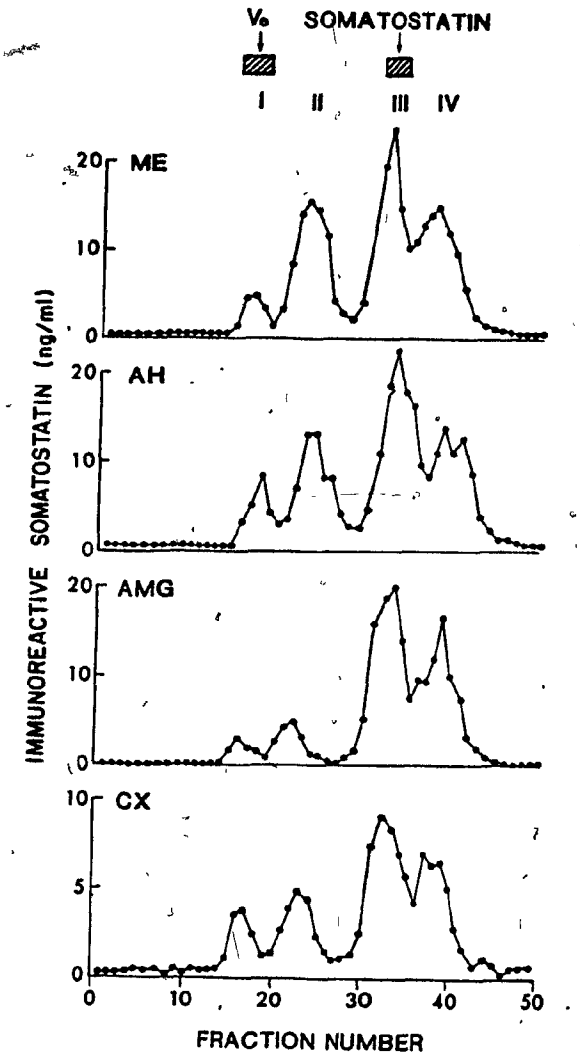


Figure 22. Gel filtration chromatography of affinity-purified IRS from rat brain. The column size was 1.1 × 50 cm, the eluent was 0.2 M acetic acid containing 0.1% HSA (Cohn fraction V), the flow rate was 12 ml/h, the temperature was 4°C and the fraction size was 1.45 ml. The void volume (Vo: determined by blue dextran and ^{125}I-labelled rGH) and the elution position of synthetic somatostatin are indicated.

to that of synthetic somatostatin. An equal aliquot of the four peaks from each brain region was neutralized with one eighth the volume of NaOH and tested for its ability to inhibit the release of rat GH. The dose of IRS from each peak was not standardized but was proportional to the quantity of IRS shown in each peak in Figure 22. The IRS from all gel filtration peaks of the four brain regions significantly ($p<0.02$, by two-tailed t test) inhibited the release of rat GH from dispersed anterior pituitary cells compared to controls (Figures 23 A and B)

Figures 23 A and B. Release of rat GH from cultured rat anterior pituitary cells in the presence of IRS peaks (I,II,III,IV) separated on gel filtration chromatography as shown in Fig. 22. Samples tested were selected from the elution fractions of each peak which contained the maximum concentration of IRS. The two panels refer to different bioassays. Levels of significance are determined by a two tailed t-test. The number of incubation dishes used to determine the mean release of rat GH are indicated. The vertical lines represent the SEM.

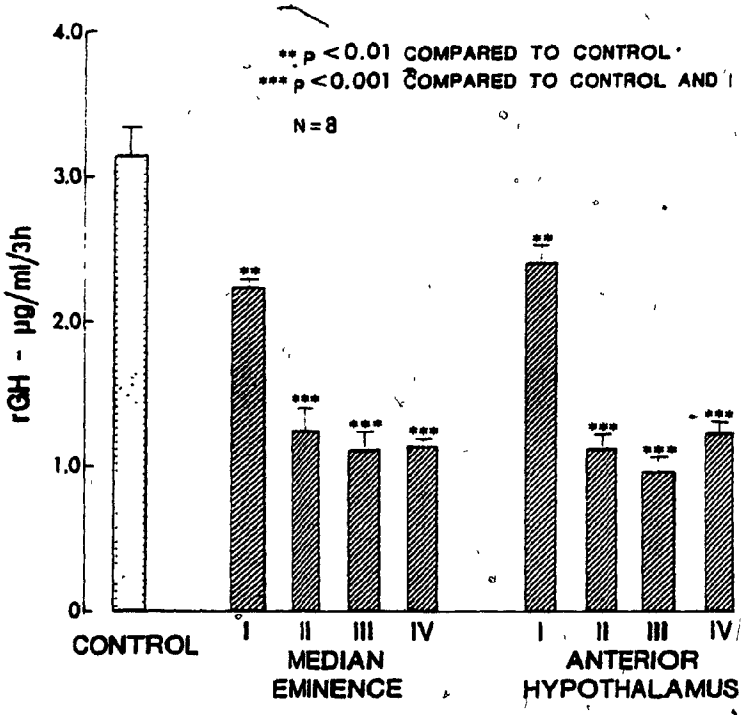


Figure 23 A

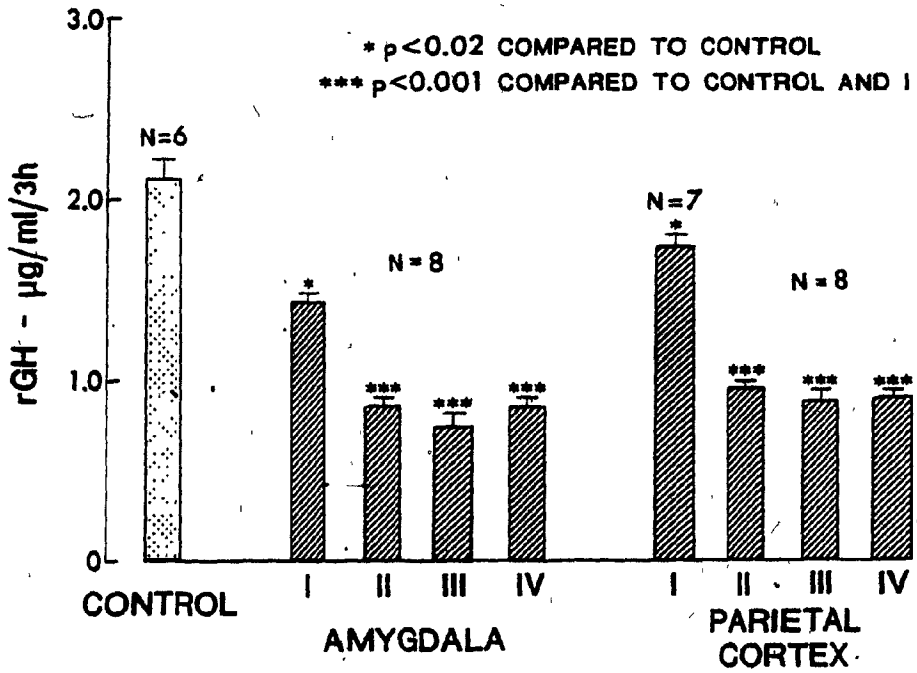


Figure 23 B

DISCUSSION

This thesis essentially describes the use of a SS-AS for RIA and affinity chromatography techniques and their applications to the study of somatostatin in the nervous system. The SS-AS was raised by immunizing sheep with synthetic somatostatin mixed with methylated BSA. The use of this method that does not require covalent coupling of the peptide to a larger molecule confirms the similar previous success of Epelbaum *et al* (73) using rabbits. A number of RIAs using SS-ASs raised by immunization with somatostatin covalently coupled to a larger immunogen have a lower minimum detectable concentration (Table 2) than the RIAs described herein and by Epelbaum *et al* (73). However, the experience with our method of immunization has not included a sufficiently large number of animals to conclude that it generally tends to produce a SS-AS of lesser sensitivity than the other methods listed in Table 2.

Because somatostatin contains no amino acid suitable for radioiodination we chose to use the analog [Tyr^1]somatostatin for this purpose. Studies of the stability of [^{125}I-Tyr^1]somatostatin revealed that sheep serum or, to a lesser extent, partially purified protein preparations reduced the binding of the labelled analog to SS-AS presumably by enzymatic degradation of the label (Tables 7 and 8). A similar result had previously been obtained by Patel and Reichlin (450). Use of a more highly purified protein, BSA (RIA grade), or addition of EDTA inhibited the inactivation of the label. It was advisable to store the label at -30°C because storage of the label alone at 4°C resulted in a more rapid decline of binding capacity.

Studies using somatostatin analogs determined that our antiserum recognizes the central portion of the somatostatin molecule (Tables 9 and 10). Comparison of the immunoreactivity of alanine-substituted analogs of somatostatin with the *in vitro* biological activity of these analogs (Table 2 of ref 247) reveals considerable overlap of amino acid residues essential for both binding to our antiserum and inhibition of GH release.

Acetic acid has proven a satisfactory solvent for the extraction of somatostatin from tissue and plasma in our studies and others reported

in the literature. Hydrochloric acid (0.1 mol/l) was an extraction solvent of comparable effectiveness to acetic acid providing that the extracts were assayed shortly after extraction (Table 11). During a storage time of several weeks the concentration of IRS in 0.1 mol/l HCl extracts declined considerably, rendering HCl a less suitable extractant than acetic acid. The recovery of synthetic somatostatin added to 2 mol/l acetic acid homogenates or extracts of several rat tissues was nearly complete. The tissue extracts displaced [^{125}I-Tyr^{1}]somatostatin from antiserum in a parallel manner as did synthetic somatostatin suggesting the presence in the extracts of material that was immunologically similar to somatostatin.

The concentration of IRS measured by RIA of extracts of several rat brain and gastrointestinal regions (Table 14) were comparable to those recorded in the literature (Tables 3 and 5). The hypothalamus, anterior hypothalami-preoptic area and amygdala contained the highest IRS concentrations of brain regions tested and the olfactory bulb and cerebellum had the lowest levels. The cerebral cortex, brainstem and cervical spinal cord contained intermediate IRS concentrations. The pancreas and gastric antrum had higher levels of IRS than the intestinal regions. The liver, a tissue that has not been reported to contain IRS, did not react with our RIA.

The affinity chromatography method described provided a convenient technique for concentration and partial purification of tissue and plasma IRS and for the quantitative removal of IRS from tissue extracts. It was primarily used in these studies because preliminary gel filtration studies of crude brain extracts had not produced satisfactory elution profiles. In addition, it served to remove non-IRS materials in brain extracts which could potentially interfere with the assessment of the biological activity of IRS. In the study of retinal and plasma IRS the concentrating capability of affinity chromatography proved of considerable utility. Two observations support the conclusion that the IRS recognized by our antiserum has biological activity. First, rat brain IRS separated by affinity and gel filtration chromatography inhibited the release of GH from rat anterior pituitary cells (Figure 23). Second, subtraction of IRS from an extract of porcine SME by affinity chromatography greatly reduced

the GH release-inhibiting activity of the residual extract (Table 15). Whether removal of IRS from tissue extracts by affinity chromatography will be of assistance in the investigation of GH releasing factors remains to be determined.

The rat retina contained a material that exhibited immunological, biological, and chromatographic properties similar to those of somatostatin. The affinity chromatography experiments demonstrated that retinal IRS bound reversibly to immobilized antisomatostatin immunoglobulin. The IRS that had thus been partially purified and concentrated retained the capacity to compete with [^{125}I-Tyr^{1}]somatostatin for antiserum binding in the RIA and to inhibit the release of GH from rat anterior pituitary cells in a fashion comparable to that of crude retinal extracts and somatostatin. Gel filtration chromatography of the immunoaffinity-purified rat retinal IRS resulted in a predominant peak of IRS that eluted at a similar volume as did synthetic somatostatin and a smaller peak that eluted at the column void volume. The concentration of IRS in the retina was less than that reported for the majority of rat brain regions, including the hypothalamus, amygdala, cerebral cortex, brainstem and spinal cord (Table 14). Rat brain areas that contain levels of IRS comparable to that found in the retina are: cerebellum and olfactory bulb (Table 14), pineal (Table 3) and substantia nigra (90,91).

It was of interest that the ratio of bioassayable somatostatin to IRS determined on crude retinal extracts was above unity. This taken together with the fact that the bioassay/RIA ratio of somatostatin purified by affinity chromatography was less than unity, suggests the presence of material in the crude rat retinal extract that inhibits the release of GH but does not react with our SS-AS. Both reports of the initial purification of somatostatin from sheep and porcine hypothalami contain evidence of preparative fractions with GH release-inhibiting activity that are distinct from the fractions from which somatostatin was isolated (5, 57). Moreover, Vale *et al* (72) have studied extrahypothalamic rat brain by bioassay and three different RIAs and have observed bioassay/RIA ratios of 1.26, 2.16, and 1.86*.

*Calculated from data in Table 1 of reference 72.

Our studies did not determine in which cellular elements of the retina somatostatin-like material is localized. The fact that IRS content was preserved in experimental models of photoreceptor and ganglion cell degeneration argues against localization in these cells. The optic nerve transection data also indicate that the retinal content of IRS is independent of central connections. This observation is consistent with either local biosynthesis or uptake of IRS from blood or vitreous humor.

Other workers have recently reported the presence of IRS in the retina of rats (460), frogs and goldfish (461) and a tissue piece consisting of the rat sclera, choroid and retina (462). Elde *et al* (463) observed by immunohistochemical methods IRS-positive nerve fibers and varicosities in the rat retina in a lamina corresponding to the junction of the inner nuclear and inner plexiform layers. IRS-positive amacrine cells were identified in studies of the goldfish retina (461). The amacrine cell has its cell body situated in the inner nuclear layer of the retina and forms horizontal connections predominantly in the inner plexiform layer. Its function is incompletely understood. In addition to IRS, immunoreactive enkephalins have been localized to amacrine cells of the pigeon and chicken retinas (464). Immunoreactive substance P (465), TRH (466) and enkephalins (467) have also been identified in extracts of the rabbit, rat and chicken retinas. Considering the likelihood of the retina containing several biological active peptides, it may prove a useful model tissue for future studies of the relationship between peptides and neuronal function.

Preliminary studies of human retinal extracts revealed the presence of IRS that behaved on affinity chromatography in a similar manner as did rat retinal and brain IRS. Human retinal and hypothalamic extracts showed GH release inhibiting activity in the bioassay. All extracts of retinas from 11 humans contained IRS. However, the concentration of IRS appeared to fall with storage time in HCl, a result that is consistent with similar data obtained with rat brain extracts. Therefore, a mean concentration of human retinal IRS has not been given because the experimental samples were stored for different periods of time. The concentrations of IRS in the retinal extracts that were stored for the shortest periods of

time approximate the true human retinal IRS concentrations more closely than the other samples.

Evidence from the literature has been presented above for the existence of an IRS-containing neuronal projection from cell bodies of the anterior hypothalamic-preoptic area to the ME. The demonstration by Gainer and colleagues (468) that neurophysin undergoes modification from a large to a smaller molecular weight species during axonal transport from the supraoptic nucleus to the neurohypophysis provoked interest in the question of whether a similar mechanism may function in the IRS-containing projection from the anterior hypothalamus-preoptic area to the ME. Accordingly, we chose to examine the chromatographic and biological properties of IRS extracted from the rat ME, anterior hypothalamus-preoptic area and extrahypothalamic brain regions represented by the amygdala and parietal cortex.

Extracts of IRS from the rat ME, anterior hypothalamic-preoptic area, amygdala and cerebral cortex behaved in a qualitatively similar manner on affinity and gel filtration chromatography. In the case of the ME and anterior hypothalamic-preoptic area, this observation suggests one of the following alternatives: 1) that IRS synthesized in the anterior hypothalamic-preoptic area cell bodies is transported by axons to nerve terminals in the ME with no structural modification, 2) that if transport-associated chemical modification occurs, it could not be detected by the methods used, or 3) that transport of IRS does not occur between the anterior hypothalamic-preoptic area and the ME. In the latter case, the dependence of the content of IRS in the ME on the integrity of neuronal connections from the anterior hypothalamic-preoptic area would have to be due to an as yet undefined mechanism, such as a transsynaptic influence upon other IRS-containing neuronal elements in the ME.

The demonstration of more than one peak of IRS on gel filtration of the affinity-concentrated rat brain extracts is consistent with the heterogeneity of tissue IRS detailed in the introduction. However, it must be emphasized that the gel filtration conditions used in the present study do not rule out the possibility that the observed heterogeneity of brain IRS may be due to noncovalent aggregation of somatostatin, binding to

larger molecules, or the presence of dimers, trimers, or larger complexes of somatostatin connected by interchain disulfide bonds. Therefore, a valid estimate of molecular size cannot be given for the peaks identified; further study is required for their determination. However, the peaks identified appear common to all four brain regions studied. The fourth peak of IRS that eluted in a position after synthetic somatostatin is of interest because of its retardation on sephadex. This may indicate that it represents a form of IRS that is more hydrophobic than somatostatin. Retardation of hydrophobic molecules on sephadex chromatography has been previously described (469). Others have observed an IRS peak eluting later than synthetic somatostatin on gel filtration of extracts of sheep hypothalamus (134), rat pancreas (226) and a human somatostatinoma (230).

At present it is unclear why gel filtration chromatography of the retinal extract resulted in predominantly one peak of IRS. The retinal experiments differed somewhat from the others; the retinas were extracted in 0.1 mol/l hydrochloric acid whereas the other brain regions were extracted with acetic acid. Further investigation will be required to determine if the different chromatographic profiles of the retina and other brain regions are due to methodological factors or truely reflect lower concentrations of heterogeneous forms of IRS in the retina.

Heterogeneity of polypeptide hormones with respect to molecular size, biological activity, and immunoreactivity is more the rule than the exception, including insulin, parathyroid hormone, gastrin, ACTH, calcitonin, glucagon, cholecystokinin and somatostatin among others (470-474). Heterogeneity may be due to the identification by RIA of biosynthetic precursors, chemically modified forms or products of proteolytic cleavage of the hormone. For instance, prohormone forms have been identified for insulin (475), glucagon (476), calcitonin (477), parathyroid hormone (478), neurophysin (468) and somatostatin (231). Heterogeneous forms of a hormone may have different degrees of biological activity, for instance the forms of gastrin (471) and the large molecular weight forms of glucagon-like polypeptide from the pig colon (479). The transformation of one form of a polypeptide hormone to another form with a

different biological potency may reflect a control mechanism that modulates the expression of a hormone's biological activity.

Each peak of IRS identified by gel filtration chromatography of all rat brain regions in Figure 22 significantly inhibited the release of GH from cultured rat anterior pituitary cells. Although peak I caused less inhibition of GH release than the other peaks, this does not necessarily imply a lower biological activity of the IRS found in this peak because the concentration of IRS in peak I was lower than that in the others. Bioassayable GH release-inhibiting activity had previously been observed by Krulich *et al* (62) in the rat anterior hypothalamus and ME and by Vale *et al* (92), in several rat brain regions. In addition, Spiess *et al* (224) noted that a peak of IRS from rat hypothalamus which eluted earlier than synthetic somatostatin on gel filtration chromatography was biologically active. Considering that the RIA and bioassay used in this study both recognize the central region of somatostatin, presumably the somatostatin-like materials in rat brain must contain a region structurally similar or identical to the interior portion of the somatostatin molecule which accounts for the expression of immunological and biological activity.

In conclusion, IRS extracted from four hypothalamic and extrahypothalamic rat brain regions exhibited similar chromatographic and biological properties, although the gel filtration behavior of IRS from each region was complex. These similarities among regional IRS render unlikely the possibility that extrahypothalamic somatostatin-like material in the rat brain represents merely an immunologically cross-reactive substance unrelated to somatostatin.

APPENDIX

Table 20. Statistical analysis of linear regression lines of binding of [^{125}I-Tyr^{1}]somatostatin to antiserum as a function of the concentration of somatostatin or tissue extract. First the residual variances about the regression lines were compared by a two-tailed *F* test as described by Snedecor and Cochran (480) with a null hypothesis of no difference between the residual variances. Second, the slopes of two regression lines were tested for parallelism by a two-tailed *t* test described by Dixon and Massey (481) with a null hypothesis of no difference between the slopes. The residual variances about the regression lines of the rat tissue extracts and somatostatin were not different ($p>0.05$). The residual variances for the human hypothalamus and retina lines were different ($p<0.05$) from that of the somatostatin line but the significance of this is uncertain because of the small sample size. The slopes of the regression lines for somatostatin were not different ($p>0.1$) from those of both rat and human tissue extracts tested.

Table 20.

somatostatin line compared to:	Residual variance about regression line		slope	
	F	*p*	*t*	*p*
rat hypothalamus	1.15	>0.05	0.026	>0.1
rat amygdala	1.57	>0.05	0.099	>0.1
rat cerebral cortex	23	>0.05	0.036	>0.1
rat pancreas	2.15	>0.05	0.587	>0.1
rat stomach	2.34	>0.05	0.483	>0.1
rat duodenum	1.41	>0.05	0.602	>0.1
rat jejunum	1.21	>0.05	0.136	>0.1
rat colon	3.70	>0.05	0.667	>0.1
rat retina	1.72	>0.05	0.491	>0.1
human hypothalamus	42.3	<0.05	0.072	>0.1
human retina	137	<0.05	0.133	>0.1

STATEMENT OF ORIGINALITY

The RIA for somatostatin used for these studies is the only example of a somatostatin RIA using a sheep antiserum induced by the method described herein. Drs. Epelbaum, Brazeau and Benoit collaborated in the development of portions of this RIA and have used it in subsequent studies of their own. The application of affinity chromatography for the demonstration of IRS in plasma is original and the use of affinity chromatography for study of IRS in brain was original at the time the studies were done.

The combination of RIA, bioassay and affinity chromatography for the study of IRS in brain, including the retina, is original. Somatostatin-like activity in retina had not previously been described. The particular experimental protocol used for the study of chromatographic and biological properties of brain IRS has not been described by others. Dr. M.J. Brownstein collaborated in the determination of IRS content in the presence of retinal degeneration. Drs. K. Hoyte and M. Senterman collaborated on the collection of human retinas. Dr. J. Epelbaum collaborated in the early stages of the experiments described in Section E.

The studies described herein have appeared in the following original abstracts and articles.

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