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REDUCED FORMS OF SOMATOSTATIN IN PERIPHERAL TISSUES OF THE GUINEA PIG

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Reduced Forms of Somatostatin in Peripheral Tissues of the Guinea Pig", submitted by James Stuart Williams in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Peripheral tissues of six guinea pigs, including pancreas, antrum of stomach, duodenum, ileum, and colon, were homogenized and extracted in hot acetic acid. The homogenates were purified to yield a clear peptide extract which was further fractionated by high performance liquid chromatography. The fractions thus obtained were assayed for somatostatin-like immunoreactivity (SLI). All tissues examined exhibited SLI corresponding to somatostatin-14 and -28, as well as late somatostatin. In addition, SLI was found in two peaks corresponding to dihydrosomatostatin-14 and -28. These findings are novel in that this is the first identification of reduced forms of somatostatin in pancreatic and gastrointestinal tissues, and also in that this is the first clear demonstration of dihydrosomatostatin-28 in any tissue.

Total somatostatin-like immunoreactivity in the tissues ranged from 16.2 pmol/mg protein in the ileum to 82.5 pmol/mg protein in pancreas. Somatostatin-28 represented between 7% and 41% of total SLI in the tissues; somatostatin-14 represented between 38% and 67% of total immunoreactivity. Of the reduced forms, dihydrosomatostatin-14 represented 2% to 12% of total SLI in the five tissues, and dihydrosomatostatin-28 represented between 6% and 15% of total SLI.

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DEDICATION

This thesis is dedicated to Dr. Nancy Henderson:
a superior teacher, human being, and friend.

TABLE OF CONTENTS

Chapter

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

H_2-S14	dihydrosomatostatin-14
H_2-S28	dihydrosomatostatin-28
HPLC	high performance liquid chromatography
preproS	preprosomatostatin
proS	prosomatostatin
RIA	radioimmunoassay
RP-HPLC	reverse-phase high performance liquid chromatography
S14	somatostatin-14
S20	somatostatin-20
S25	somatostatin-25
S28	somatostatin-28
SLI	somatostatin-like immunoreactivity
DTT	dithiothreitol
BSA	bovine serum albumin
TFA	trifluoroacetic acid
HFBA	heptafluorobutyric acid

I. Introduction

Somatostatin, also known as somatotropin release inhibiting factor (SRIF), was originally identified by Krulich and associates (1) more or less incidentally while they were engaged in a search for the hypothalamic factor responsible for stimulation of growth hormone release from the anterior pituitary. The discovery of somatostatin led them to hypothesize the interaction of two regulatory neurohormonal factors: one inhibitory, the other stimulatory. A pancreatic factor similar to their hypothalamic factor was found by Hellman and Lernmark at close to the same time, which they determined had the effect of inhibiting insulin secretion (4). These two factors appeared to be unrelated until the elucidation of the primary structure of hypothalamic somatostatin by Guilleman's laboratory in 1973 (2); it was soon apparent that the pancreatic and hypothalamic hormones were one and the same tetradecapeptide (3).

These discoveries opened the floodgates, and since that time a staggering volume of research has been described in the literature of the somatostatin-like peptides. The term 'somatostatin' is now recognized as something of a misnomer, for the range of physiological functions of somatostatin is such that a name that describes its growth hormone-inhibiting role alone is misleading.

Given the enormity of the somatostatin literature, this review must necessarily be limited in depth. Nonetheless, those topics that pertain to this research project will be dealt with in greater detail.

A. Distribution of Somatostatin

1. Phylogeny

Somatostatin-like immunoreactivity (SLI) has been described in tissue extracts from members of all vertebrate classes, from the sea squirt (a protochordate), and from at least one invertebrate species (4). In addition, SLI has been detected in a protozoan and in two species of bacteria, suggesting that the peptide antedates the development of multicellular organisms and their need for intercellular communication mechanisms (3,5).

Most recently, SLI has been located in two species of flowering plants; this immunoreactivity corresponds to two peaks on HPLC analysis corresponding to somatostatin-28 (S28) and somatostatin-14 (S14) (5). Bacterial, protozoan, and plant somatostatins have all been shown to exhibit biological activity in mammalian tissue bioassays. The presence of these peptides across such a broad phylogenetic range argues for a critical and basic need for somatostatin to ensure species success in nature.

2. Interspecific Variation in Somatostatin Primary Structure

The primary structure of somatostatin in all vertebrate classes so far examined is quite highly conserved. Among mammals it is invariant, whether from normal or tumoral tissue; this applies to both S14 and S28 (4,6). Comparison of vertebrate somatostatins shows a high degree of conservation (see Figure I). Comparison of cDNA sequences for Anglerfish I and rat thyroid somatostatins shows 83% nucleotide homology for S14 sequences and 69% for S28 sequences.

Amino acid conservation is 79% for S28, and the six substitutions are sterically and functionally conservative; S14 is completely conserved. N-terminally extended forms of somatostatin (preproS, proS, $proS_{33-92}$, and $proS_{33-76}$; see Figure 2) are less highly conserved than S14 and S28, although critical residues such as Glu-32/Leu-33 and Asn-61/Gln-62/Thr-63 exhibit degrees of conservation comparable with the sequences of the biologically active hormones (56).

3. Anatomical Distribution and Molecular Heterogeneity

Immunoreactive somatostatin has been described in an abundance of mammalian tissues. Centrally, SLI has been localized to the hypothalamus, especially the paraventricular nucleus; somatostatinergic terminals in the median eminence function in regulation of the anterior pituitary. Somatostatinergic neurons also terminate in the neurohypophysis and may modulate arginine vasopressin and oxytocin secretion. They are also found in the cerebral cortex, hippocampus, amygdala, and project into the brain stem and spinal cord. There appear to be somatostatinergic fibers intrinsic to the spinal cord as well (4,7,8).

In peripheral nervous tissues, SLI has been described in sensory and autonomic ganglia (4), portions of the auditory nerve and cochlea (9), and retina (10,11). Intrinsic somatostatinergic neuronal elements have also been identified in gastrointestinal tissues (17).

Non-neural peripheral tissues containing SLI include pancreas, gastric antrum and fundus, and intestinal mucosa and muscle wall, primarily (4,12). Immunoreactive somatostatin has been localized to

the salivary glands and their exocrine secretions (13), thyroid parafollicular cells (14), and in amphibian and mammalian kidney (including man) (4,15). Visceral SLI appears to be predominantly attributable to cells typified by D-cells of the endocrine pancreas, which act via paracrine and probably endocrine modes; the remainder is present in somatostatinergic neurons, both intrinsic and extrinsic to gut tissue (3,4,16,17).

Considerable effort has gone into elucidation of the primary sequences of the peptides comprising the total SLI in immunoreactive tissues, and considerable heterogeneity has been noted. This heterogeneity appears to adhere to a general scheme described by Patel and associates, who first noted three major patterns in the rat. These patterns include the brain pattern (70-75% S14, 30%S28 and proS), pancreatic pattern (90-95% S14, 5-10% S28 and proS), and gastrointestinal mucosal pattern (60% S28, 22% S14) (17).

Mammalian tissues that follow the brain pattern, within tolerable limits, include gastric D-cells (18,20,22), as well as most central nervous system structures and peripheral somatostatinergic neural tissue (4,17,18,20). The pancreatic pattern holds true for all mammalian and avian pancreatic islets except those of the guinea pig, where >99% of SLI is ascribed to S14 (21). The mucosal pattern has been found to be the norm in all mammalian gut tissues examined (4,17,18,20). An interesting deviation from these three patterns is seen in some mammalian retinas: most species exhibit heterogeneity following brain pattern, but roughly 60% of bovine and guinea pig retina SLI is ascribed to S28 (10,20). As other tissues and species

are assayed it will be seen whether these apparently standard patterns remain valid across the phylogenetic spectrum, especially as techniques capable of finer resolution of peptides become available.

In addition to that attributed to S14, S28, and proS, somatostatin-like immunoreactivity has been ascribed to other--albeit related--peptides. Somatostatin-28_{1-12} is abundant in the CNS and digestive systems of rodents and primates; concentrations of this peptide are comparable to those of S14, leading to the hypothesis that it is not a physiologically relevant hormone, but is rather a byproduct of peptide processing (4,7,26). Somatostatin-25, an N-terminally extended form of S14, has been isolated from hypothalamic and gastrointestinal extracts; this peptide exhibits significant biological activity, but results reported in the literature are discrepant and physiological relevance remains unproven (27,28). A third and novel peptide with biological activity, somatostatin-20, has been isolated from porcine duodenum, but it is also of questionable physiological significance (28).

In addition, dihydrosomatostatin (H_2-S14) has been isolated from guinea pig brain in significant quantities, although a biological role for this peptide has yet to be established (29). High resolution techniques (HPLC and RIA following gel filtration of extracts) have revealed that reduced somatostatin is not present in ground squirrel brain(19), in guinea pig retina(11), or in rat brain regions (hypothalamus, cerebellum, cortex, brainstem, amygdala, and neurohypophysis) (94-96). Additionally, human brains (cortex) subjected to extraction and subsequent analysis exhibited S14, S28,

and proS immunoreactivity, but no reduced peptides (97). The function of reduced somatostatin in the guinea pig is an interesting topic for investigation, given the lack of evidence for this factor in any other species.

B. Physiology of Somatostatin

1. Mechanisms of Secretion

Considering the number of physiological functions reputedly regulated by somatostatin, it should not be surprising to find a correspondingly large number of factors affecting its secretion. As is the case in most secretory cells, secretion of somatostatin is associated with cell membrane events including depolarization (sodium and potassium ion flux) and Ca^{2+} flux. The first stimulus of S14 secretion identified in the hypothalamus (and in pancreatic D-cells) was initiated by electrical pulses; this effect has been duplicated by other standard depolarizing conditions such as elevating extracellular K^{+} levels or adding ouabain or veratridine to the incubation medium (7). Similarly, sodium channel blockade has been shown to prevent somatostatin release from axon terminals (4). Release of SLI from synaptic terminals of somatostatinergic neurons and by D-cells is calcium-dependent; low calcium ion concentrations in extracellular fluid and blockade of Ca^{2+} influx by verapamil both serve to inhibit secretion of the peptide (4,7,66,67). Islet-activating protein (IAP) from Bordatella pertussis has been shown to markedly augment somatostatin release from D-cells in culture; since IAP has been clearly shown to induce rapid increases in cytosolic

Ca^{2+} concentrations (89), it is probable that the secretory mechanism involves increased influx of calcium ions from extracellular fluid (69,70).

Stimulatory neurotransmitters involved in regulation of somatostatinergic secretion in central and peripheral nervous tissue include dopamine, norepinephrine (in hypothalamus only), and possibly acetylcholine, serotonin, and gamma-amino butyric acid. Serotonin and acetylcholine have also been shown to have inhibitory effects in some systems (4). Gastrointestinal D-cells appear to be stimulated by inhibition of α-adrenergic stimuli, suggesting a depressive or modulating role for sympathetic neuro- transmitters in the gut (68).

Hormonal secretagogues of somatostatin include glucagon, substance P, gastrointestinal polypeptide (GIP), pancreatic polypeptide, neurotensin, cholecystokinin-4 and -8, and bombesin. Vasoactive intestinal peptide (VIP), secretin, and endogenous opiates have all demonstrated inhibitory effects on somatostatin secretion (4,7,47). Histamine-H_2 receptors have been implicated in postprandial visceral somatostatin regulation in man (47), and prostaglandins have produced mixed results: they have no apparent effect on hypothalamic secretion *in vitro*, but they stimulate gastric and pancreatic elements in culture and *in vivo* (40,47).

Correlation between circulating nutrient levels and somatostatin secretion has also been demonstrated, as would be expected if it is a true regulator of nutrient homeostasis. Glucose, amino acids, and free fatty acids (but not triacylglycerols) have been shown to increase somatostatin secretion from isolated and perfused rat and

dog pancreas, and from rat pancreas in vivo; gastric secretion in rats, however, does not appear to respond to circulating nutrient levels (4,47).

Somatostatin is quite hydrophilic in character, so it is readily transported once released into blood or interstitial fluid without the aid of specific plasma carrier or binding proteins.

2. Somatostatin Receptors

High-affinity receptors for somatostatin have been identified in a number of tissues, including brain regions, pituitary, pancreatic endocrine and exocrine elements, and gastrointestinal tract structures; in addition, receptors have been characterized on pituitary GH_4C_1 and murine AtT-20 tumor line cells (4,7,42,71,72). Receptor characterization has been hampered by the requirement for tyrosine-substituted radiolabelled analogs due to the lack of tyrosine residues in native somatostatin: as a result, there is no standard analog accepted and used by all laboratories, and even if a common standard analog was to be agreed upon, it is not at all certain that the binding characteristics of any [Tyr]-somatostatin analog are anything more than approximations of those of the native peptide. Further, it appears that degradation of the analogs by membrane preparations varies depending on the position of the [Tyr] substitution; since stability of the radioligand must impact on the observed binding parameters, it seems that we are not only faced with diverse affinity constants in analog binding experiments, but that differences in efficacy among analogs must also be assumed to exist.

Perhaps one of the most serious problems--but one that is

relatively simple to correct, and, once corrected, likely to yield great insight into the receptor--in somatostatin receptor studies to date is that heterogeneous cell populations have been used in binding experiments. This fact introduces the possibility that more than one receptor type may exist, consequently reported binding characteristics may actually be hybridized, reflecting the characteristics of two distinct receptor subtypes (42).

Pituitary S14 receptors have shown insensitivity to the presence of calcium ions, unlike pancreatic and gut receptors (7,72). Most peripheral tissues, as well as brain, exhibit higher affinity for S14 than S28, and some laboratories claim to have identified distinct receptor populations that preferentially bind one form or the other in normal and tumoral tissues. Autoradiography in vivo has also indicated preferential S14 and S28 binding sites in the median eminence and circumventricular organs (3,7,73-75). These findings in sum tend to suggest the existence of at least two subtypes of somatostatin receptor.

Down-regulation of somatostatin receptors has been demonstrated in AtT-20 tumor cells pre-incubated with S14 or S28, leading to attenuation of somatostatin inhibition of target cell activity (72,75-77). Decreased receptor affinity for ligand has also been reported by some investigators (76), but ruled out by others (77).

At present it seems that the status of somatostatin receptors is an uncertain quantity, although the information pool is growing rapidly.

3. Post-Receptor Cellular Regulation

The effects of somatostatin within target cells may be mediated by two separate mechanisms, the first cAMP-dependent, the second cAMP-independent.

Several types of studies support the hypothesis that somatostatin inhibits pituitary hormone release by depressing intracellular cAMP levels: first, cAMP analogs and cAMP phosphodiesterase inhibitors increase secretion of growth hormone, thyrotropin, and prolactin by pituitary cells in culture; second, S14 will inhibit basal and stimulated cAMP synthesis under certain conditions in these same cell lines; and third, S14 inhibits PGE_1-stimulated adenylate cyclase activity in GH_1 cells (78). Further, guanine nucleotide-binding proteins (specifically, G_i) have been implicated in the transduction of somatostatin receptor-mediated inhibition of adenylate cylase: addition of exogenous GTP significantly reduces somatostatin receptor affinity for ligand (78), and pertussis toxin (which ADP-ribosylates G_i, rendering it incapable of binding guanine nucleotides) has been shown to abolish somatostatin inhibition of cAMP accumulation in AtT-20 tumor cells(79).

A cAMP-independent mechanism for cellular regulation by somatostatin involving calcium-dependent entities has also begun to emerge. Somatostatin transiently reduces free Ca^{2+} in GH_3 cell cytosol (78); similarly, it simultaneously blocks adrenocorticotropin release and calcium mobilization as measured by Quin-2 fluorescence in stimulated AtT-20 cells (79). Somatostatin has been shown to inhibit prostaglandin E_1- and theophylline-induced salt and water

secretion by gut mucosal cells without affecting cAMP accumulation, suggesting that it can act downstream of the cyclic nucleotide (50). The nature of this mechanism is unknown, but G-proteins may again be involved. This is suggested by the fact that pertussis toxin blocks S14 inhibition of calcium mobilization (78,79). It has been suggested that blockade of calcium mobilization.is a secondary effect of somatostatin's ability to depolarize membranes by increasing permeability to potassium (80), but this hypothesis remains untested.

One interesting and potentially major mechanism by which somatostatin may regulate cellular events is by activation of phosphoprotein phosphatases in the cytosol; this would effectively inhibit activity of both cAMP-dependent and calcium-dependent protein kinases, as well as their multitude of phosphoprotein substrates (81). However, somatostatin activation of phosphatase has never been successfully reproduced by laboratories other than the original authors', so its significance as a cell regulation pathway is suspect, however attractive the hypothesis might be.

4. Biological Effects of Somatostatin: CNS

Somatostatin secreted by somatostatinergic terminals in the median eminence plays a vital role in regulating anterior pituitary hormones. The peptide inhibits basal and stimulated GH and TSH secretion, basal prolactin secretion, GH and prolactin secretion from GH_4C_1 tumor cells, and ACTH secretion from murine (AtT-20) and human cell lines (7,17,21,41). Not surprisingly, pituitary somatotrophs are more sensitive to both S14 and S28 than are thyrotrophs (7). Several researchers have noted that S28, S25, and S20 appear to be

considerably more potent than S14 in inhibition of pituitary and other CNS structures (7,17,21,41). This has led to speculation that S14 is not the fully biological active peptide, but is only a fragment of the true hormone possessing residual biological activity, and S28 has therefore been pushed forward as the "true" native form of somatostatin. By way of contrast, experiments by Schonbrunn and associates (42) have shown S14 to be much more potent than the N-terminally extended peptides. This group has suggested that the discrepancies in reported receptor affinities and biological activities of S28, and S25 as opposed to S14 may be due to the use of nonstandardized buffers and incubation media as well as use of non-homogeneous pituitary cell populations in bioassays. They have also speculated that S28 may appear to be more potent by virtue of its greater resistance to degradation; in their assays, where degradation was minimized, S28 and S25 exhibited only 30% of the activity of S14 in inhibiting cAMP acculumlation in stimulated GH_4C_1 cell lines. The evidence offered by this group cannot be safely generalized to all bioassay systems and cell lines, but it does present an attractive and simple explanation for the range of apparently discrepant reports of relative potencies of S28 and S14. It remains to be seen whether further work will support their hypotheses.

5. Biological Effects of Somatostatin: Pancreas and Gastrointestinal Tract

More than one reviewer has stated that somatostatin is involved in the regulation of virtually every aspect of visceral function. It was first thought that somatostatin's action in GI system regulation

followed paracrine pathways, but a growing body of evidence argues for acceptance of an endocrine role as well in the regulation of nutrient absorption(43),gastric acid release (44), and most intestinal exocrine and endocrine secretions (47).

Pancreatic somatostatin is firmly entrenched in the literature as a regulator of circulating nutrient homeostasis by virtue of its modulation of pancreatic secretion of insulin and glucagon (4,47,49,52). Islet D-cells (somatostatin-secreting) are located within islets in close anatomical proximity to A-cells (glucagon-secreting) and B-cells (insulin-secreting). Also, extrinsic somatostatinergic neurons have been identified that terminate on all three types of islet cells. Receptor studies have demonstrated the presence of cell-surface somatostatin receptors on A-, B-, and D-cells (3,90). It is highly probable that the islets are compartmentalized to control the direction of hormonal communication: tight junctions effectively limit the surfaces of the islet cell that come into contact with a given blood supply. The cells are oriented such that they face two different capillaries, one on the arterial side, and one on the venous side. Somatostatin receptors are concentrated on the arterial side of the D-cell, while secretion appears to be localized on the venous side of the cell; thus it is probable that the D-cell's receptors do not come into contact with its own secretions (102). It is interesting to note that there are some indications that S14 and S28 may be secreted by different sub-types of the islet D-cell; if two distinct populations of cells exist, each secreting predominantly one form of SLI, it is

conceivable that differential secretion of the two forms occurs in order to fulfill two or more discrete biological objectives (53,91).

The efficacy of S14 and S28 with respect to inhibition of insulin and glucagon secretion is a matter of some controversy. Both peptides have demonstrated ability to inhibit bombesin-induced glucagon and insulin secretion (48). Islet A-cells are reported to be fifty times more sensitive to S14 than S28, while B-cells have been found to be ten times more sensitive to S28 than S14 (53). Some investigators have found S28 more potent than S14 in suppressing all pancreatic and gastrointestinal functions regulated by SLI (48,49,50), while others have found S14 uniformly more potent (3,7). These results may not in fact hold up under close scrutiny, and as more reproducible data are generated from standardized bioassays, a different--and hopefully clearer--story may emerge.

In addition to its role in regulating insulin and glucagon secretion by the endocrine pancreas, somatostatin also serves to inhibit pancreatic exocrine secretions as well (92).

The stomach is under a complex regulatory system that appears to involve somatostatin, gastrin, gastrin releasing peptide, bombesin, cholinergic neural elements, and possibly VIP and GIP as well (44). Somatostatin inhibits acid secretion by parietal cells and gastrin secretion by gastric mucosal cells (44-47). It also appears to inhibit gastric motility and emptying (45).

Absorption of glucose, xylose, calcium ions, amino acids, and lipids by the small intestine is inhibited by intravenous infusion of exogenous somatostatin (44). Administration of anti-somatostatin

serum with a meal results in depressed plasma SLI and a correlative increase in plasma triacylglycerols (47). Somatostatin secretion is responsive to nutrients present in the lumen of the gut, further suggesting a role in the regulation of nutrient uptake (45,47), and the peptide is clearly involved in inhibition of intestinal motility in man, dogs, rabbits, and guinea pigs (44,45,50). It is thought that inhibition of motility is accomplished through activation of inhibitory nerve fibers in the gut that impinge upon cholinergic innervation to smooth muscle; presumably, this inhibition involves presynaptic hyperpolarization leading to reduction of neurotransmission of the muscle fibers.

Somatostatin is implicated in the inhibition of fluid and electrolyte secretion by intestinal mucosa; long-acting analogs have been used to successfully treat severe diarrhea in patients suffering from intestinal tumors (44, 50).

There is some evidence for somatostatin acting as a satiety signal in the rat and in man (44,51), but this function is still tentative. Evidence that somatostatin functions as a "lumone" (i.e., a hormonal factor released into the GI lumen) has been reported; intraluminal SLI has been associated with inhibition of gastric acid and pepsin secretion (46).

6. Degradation of Somatostatin

The half-life of somatostatin in blood ranges from 1 to 3 minutes in mammals (3,82). Degradative activity has been described in plasma, liver, pancreas, brain, and kidney, with hepatic activity probably representing the major component (3,4,83). Brain

degradation is likely a function of synaptic clearance of neurotransmitter, and pancreatic degradation is also essentially clearance of locally released SLI. Clearance of circulating somatostatin is accomplished by plasma and hepatic enzymes. A marked transhepatic gradient is evident upon assay of blood SLI (83).

The peptidases involved in degradation in plasma and liver include both N-terminal exopeptidases and endopeptidases, the latter being responsible for breaking the ring structure and thereby eliminating biological activity (4). Exopeptidases in plasma appear to cleave alanine from the N-terminus of S14, generating an active metabolite (84).

Hepatic degradation is primarily intracellular and membrane-associated (83). Destruction of the Cys-3/Cys-14 disulfide bond occurs four times faster in S14 than S28, although both peptides are ultimately susceptible to the same degradative pathways. Hepatic N-terminal amino-peptidases have been shown to yield metabolites such as S25 and S20; this supports the contention that S25 and S20 represent degradation products of S28 rather than deliberately synthesized variant peptides. Ring degradation of S28 and S25 is kinetically indistin-guishable (85). Trypsin-like cleavage at Arg-13/Lys-14 of S28 yields significant amounts of S14 in hepatic tissue, although this pool is not destined for secretion (83).

C. Biosynthesis and Processing

The sequences of prosomatostatin (proS) and prepro-somatostatin (preproS) have been determined in a number of tissues by both amino acid sequencing and nucleic acid techniques (3,5,20,56). A single

known gene coding for preproS in mammals has been identified, but at least two genes exist in teleosts (4). Transcription and translation events in preproS biosynthesis appear to be typical of the classic peptide pattern. PreproS in mammals is a 116-residue peptide, the first 24 amino acids constituting the signal or leader sequence; this sequence is cleaved cotranslationally as the nascent peptide extrudes into the lumen of the endoplasmic reticulum (ER) (56). The molecular weight of the preprohormone as determined from mRNA is 12.7 kDa (see Fig. 2); preproS synthesized from mRNA from rat medullary thyroid carcinoma tissue and pancreatic islet tissue in cell-free translation systems is of higher molecular weight, possibly due to glycosylation of the peptide (20,56,57,58). A potential N-glycosylation site has been identified at Asn-61/Gln-62/Thr-63 of preproS (56).

ProS begins to undergo posttranslational processing shortly after cleavage of the signal sequence. Anglerfish proS-I undergoes conversion by peptidases in the ER, but proS-II and mammalian proS appear to be converted by peptidases in secretory granules (59,60). Virtually all newly synthesized proS is associated with membranes in microsomes and granules, as are converting enzymes; it is thought that prohormone and convertases are translated and packaged simultaneously (59,61). Association of peptide with membranes suggests that prohormone receptors may exist in endoplasmic reticulum, golgi, and granule membranes. Such receptors could affect processing by facilitating prohormone transfer through the cell's transport elements, targetting prohormones to secretory granules rather than lysosomes and/or augmenting association of prohormones

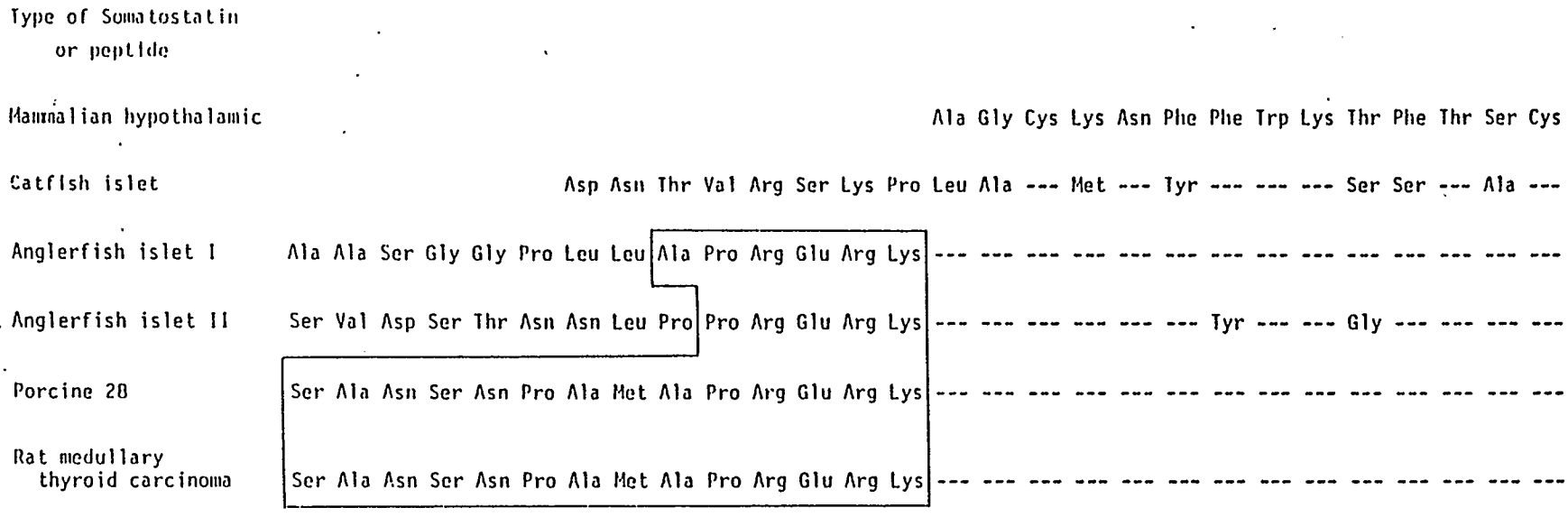

Figure 1: Primary sequence homology for six vertebrate forms of somatostatin.

NH_2-Met Leu Ser Cys Arg Leu Gln Cys Ala Leu Ala Ala Leu Cys Ile (15)
Val Leu Ala Leu Gly Gly Val Thr Gly Ala Pro Ser Asp Pro Arg (30)
Leu Arg Gln Phe Leu Gln Lys Ser Leu Ala Ala Ala Thr Gly Lys (45)
Gln Glu Leu Ala Lys Tyr Phe Leu Ala Glu Leu Leu Ser Gln Pro (60)
Asn Gln Thr Glu Asn Asp Ala Leu Gln Pro Glu Asp Leu Pro Gln (75)
Ala Ala Glu Gln Asp Glu Met Arg Leu Glu Leu Gln Arg Ser Ala (90)
Asn Ser Asn Pro Ala Met Ala Pro Arg Glu Arg Lys Ala Gly Cys (105
Lys Asn Phe Phe Trp Lys Thr Phe Thr Ser Cys-COOH (116

Figure 2: Primary sequence of preprosomatostatin indicating signal sequence (1-24), S28 (88-116), and S14 (103-116). A potential N-glycosylation site is indicated at residues 61-63. (From Goodman et al., ref. 56)

with membrane-bound converting enzymes (61).

There is considerable debate over the pattern of peptidase cleavage of proS. Some researchers have asserted that the pattern of processing is from proS to S14 via S28 as an obligatory intermediate product (62). Others have demonstrated multiple forms of immunoreactive prosomatostatin, some lacking the S14 tetradecapeptide sequence at their C-terminus; this suggests that processing may follow multiple pathways (26,58,63). Patel has published evidence showing that mammalian endocrine tissues produce S14 from S28, while neural tissues cleave S14 directly from the prohormone (99). Benoit and associates have characterized seven immunoreactive forms of somatostatin from rat brain, including N-terminally extended forms of $S28_{1-12}$ (4.4 and 7.5 kDa) and two large molecules (6 kDa and 9.5 kDa) containing the entire S28 sequence. The authors conclude from their data that a minimum of 4 cleavage sites exist in proS: at residues-14, -28, -56, and -82 (counting from the C-terminus), and that at least two identifiable peptides corresponding to all or part of proS minus the S14 sequence at their C-terminus are present. The 9.5 kDa peptide is probably $proS_{25-102}$, and the 7.5 kDa peptide is probably a fragment containing residues 57-76 (using preproS numbering; see Fig.2 and Fig.3) (64). These findings argue strongly for a model in which S14 can be generated from at least two, possibly more, large proS-like peptides in addition to generation from S28. Schmidt and associates have affirmed Benoit's findings, describing a cleavage point at Leu-56/Leu-57 in addition to the established sites at Gly-24/Ala-25 (signal cleavage), at Arg-88/Ser-89 (generating S28), and

at Lys-108/Ala-109 (generating S14) (see Fig.3) (56,58).

Cleavage at these points requires the presence of endopeptidases of high specificity. S14 is generated by a "trypsin-like" enzyme that cleaves on the COOH side of residues 101 and 102 (Arg-Lys), a fairly common peptide cleavage point. Another enzyme cleaves the C-terminal side of Arg-88, requiring that it recognize a single basic residue; and an unusual peptidase recognizes the leucine pair at positions 56 and 57. This last cleavage is not unknown elsewhere, since angiotensin I is produced from its inactive precursor by a similar cleavage (58).

It is becoming clear that the so-called trypsin-like and carboxypeptidase-like peptidases are in fact similar to their namesakes in site specificity only (61). It is probable that converting enzymes are highly specific to tissues and to species, giving rise to varied patterns of posttranslational processing of somatostatin and other peptides. For example, the processing pattern typified by hypothalamic somatostatin cells yields an unvarying mixture of S14, S28, and proS, but gut mucosal D-cells exhibit changes in processing patterns over their 5-7 day lifespan that at no time corresponds to the hypothalamic pattern (crypt cells secrete a significantly higher proportion of their SLI as S28 than do mature cells at the villus tip [25]).

Aside from proteolytic cleavages of preproS, little in the way of posttranslational modification has been identified. The potential N-glycosylation site at positions 61-63 of preproS might explain the

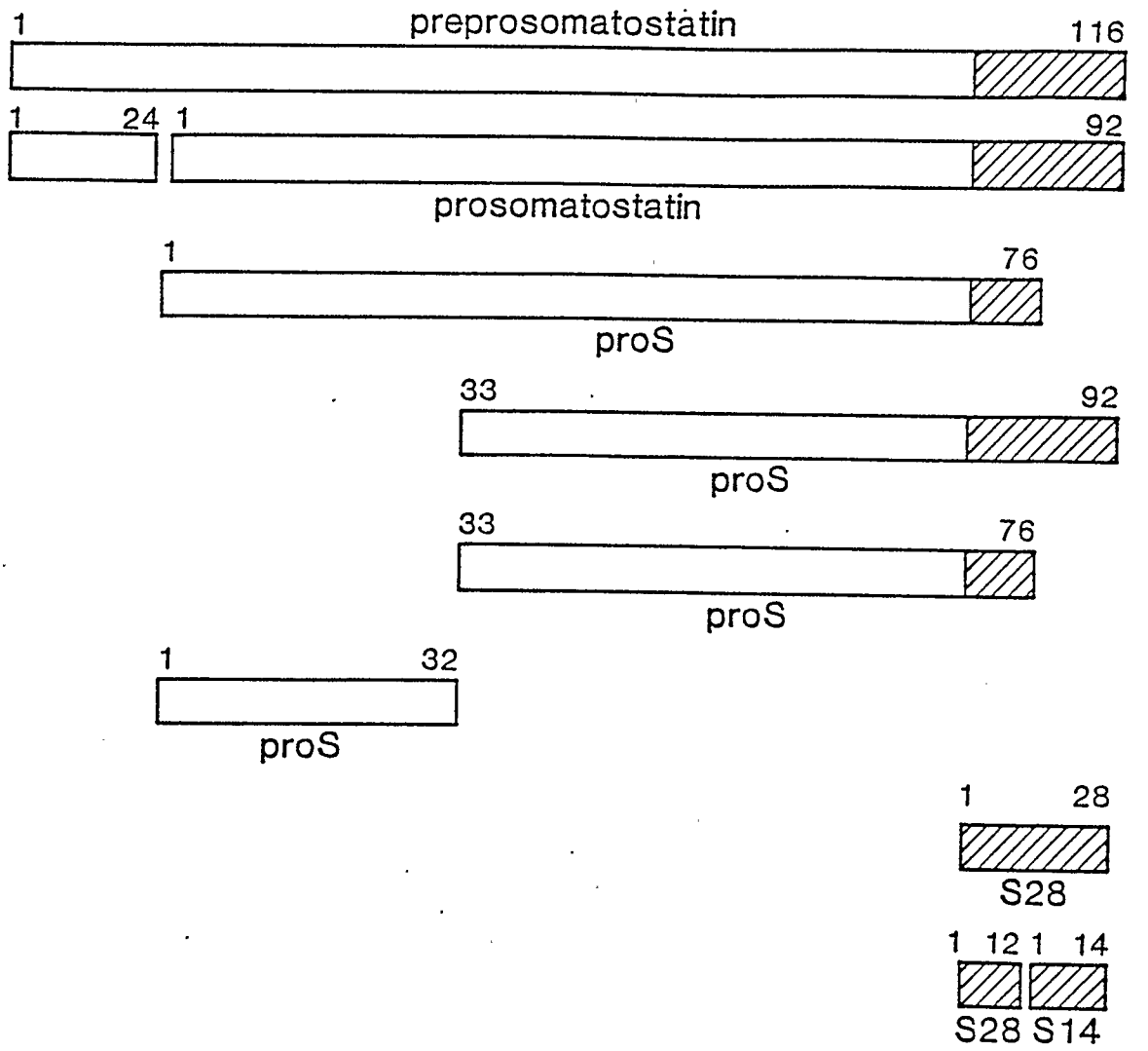

Figure 3: Schematic of preprosomatostatin processing options. Peptide fragments shown are not necessarily found in the same cell. (From Goodman, et al., ref. 56)

discrepances in molecular weight estimates for proS reported in the literature (10.4 kDa as determined by mRNA sequencing, and 12-14 kDa as determined by translation and electrophoresis [56, 57]). One other possible modification involves reduction of the disulfide bridge of somatostatin by reducing agents within the secretory apparatus of somatostatin cells (65); this hypothesis will be discussed later.

In summary, the biosynthesis and processing of preproS exhibits considerable apparent variation, both interspecific and among different tissue of the same species. These variations in processing may serve to allow the synthesis of several peptides for a multiplicity of biological ends from a single translation product, in a scaled-down manner similar to that of proopiomelanocortin (POMC).

D. Dihydrosomatostatin in the Guinea Pig

The guinea pig exhibits a number of biochemical peculiarities, including a unique progesterone-binding plasma protein, inability to synthesize ascorbic acid, severe intolerance to penicillin, a unique insulin differing from porcine standard insulin in 17 of 51 residues, and the only mammalian glucagon known to deviate from standard glucagon (22). Although neither S14 nor S28 in the guinea pig deviates from the standard mammalian form, tissue distribution is somewhat unusual (10). Evidence for the presence of a reduced form of S14 in guinea pig brain in significant concentrations adds another biochemical anomaly to the species' list; reduced (dihydro-) somatostatin (H_2-S14) was found in hypothalamus, amygdala, cerebral cortex, brainstem, and cerebellum of guinea pig brain

brain tissues (29). Extraction was performed under mildly oxidizing conditions, which suggests that the H_2-S14 peak was not an artefact of the extraction procedure; exogenous S14 added to the tissues prior to extraction increased the S14 peak but did not affect the dihydrosomatostatin peak. Treatment of the putative H_2-S14 peak with H_2O_2 and with dithiothreitol resulted in abolition and augmentation, respectively, of the peak in question, which strongly supports the assertion that the peak is in fact a reduced form of the tetradecapeptide and not another simple metabolite (see Fig. 4). .

Reduced somatostatin (H_2-S14) has been shown to have similar biological activity to S14 in bioassays <u>in vitro</u> (86,87). <u>In vivo</u>, H_2-S14 exhibits reduced biological activity but longer duration of actions (87); dihydrosomatostatin analogs that were unable to cyclize due to side chain modifications, however, exhibited very low potency (86), leading to the conclusion that the apparent activity of dihydrosomatostatin may be an artefact owing to oxidation of the peptide to S14 under bioassay conditions. This conclusion has been supported by experiments assaying dihydrosomatostatin activity in GH_4C_1 cell cultures (42).

The means by which dihydrosomatostatin is synthesized from the prohormone or from S14 in guinea pig tissues is unknown. Presumably, the disulfide bridge between Cys-3 and Cys-14 of S14 forms as the prohormone comes off the ribosome, as is normally the case in peptide or protein synthesis. In this event, a mechanism for reduction of the native disulfide bridge must exist in the endoplasmic reticulum, golgi apparatus, or secretory granules. McMartin and Purdon have

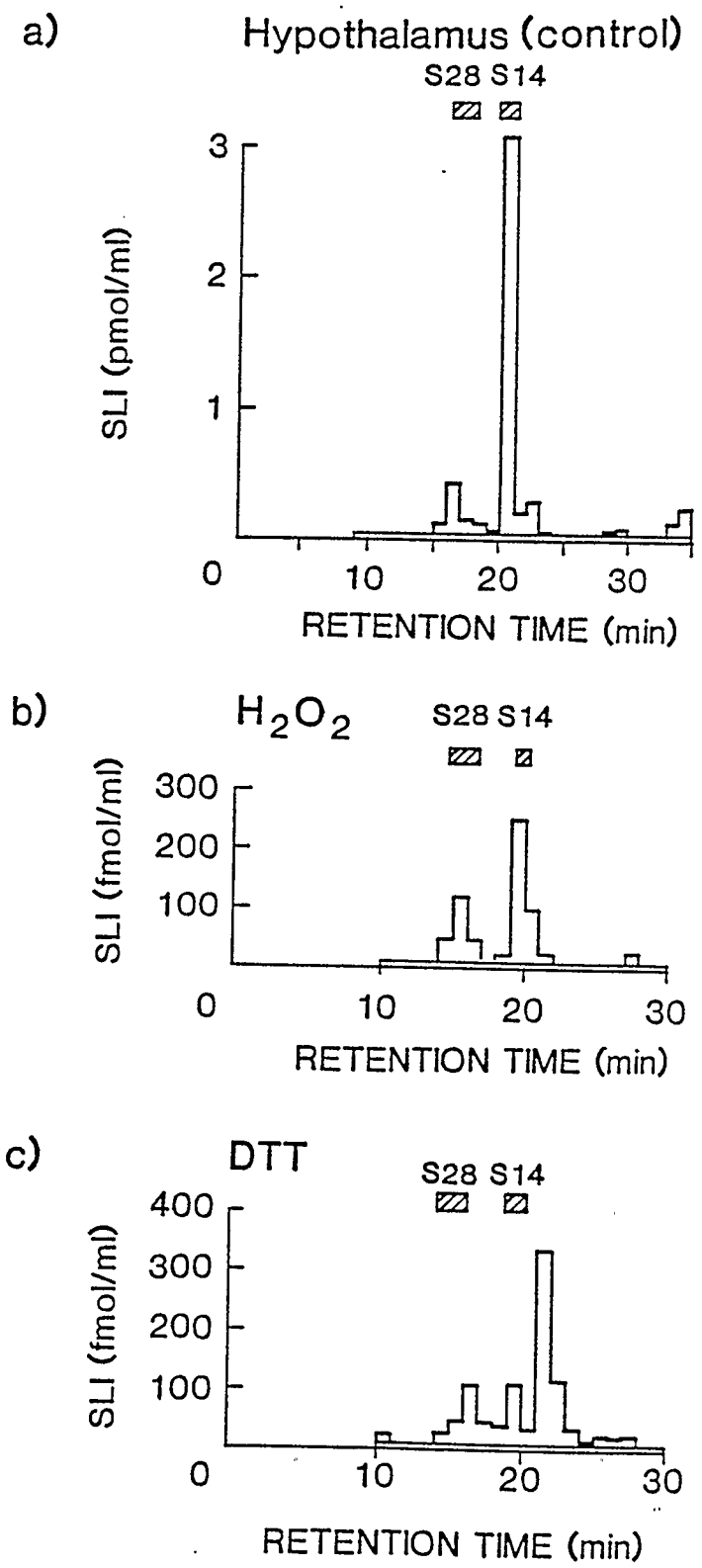

Figure 4: HPLC profiles of guinea pig hypothalamus extracts: a) control; b) incubated with H_2O_2; c) incubated with DTT. Oxidative conditions eliminated the H_2-S14 peak in control extract (RT=22-23 min), whereas reducing conditions diminished the S14 peak and enhanced the H_2-S14 peak. (From Rorstad, ref. 29)

suggested that this could involve glutathione (γ-Glu-Cys-Gly)$_2$, an entity primarily known for its role in maintaining cysteine sulfhydryl groups of hemoglobin in the reduced state (88). It is also possible that a specific reductase exists to perform the necessary function; a glutathione-protein disulfide oxidoreductase has been identified in murine mammary tissue capable of cleaving the disulfide bridges of oxytocin and insulin (112). Although this enzyme is thought to be part of the oxytocin degradation mechanism of this tissue, it is not inconceivable that such an enzyme could function in a synthetic capacity.

E. High Performance Liquid Chromatography Theory

High performance liquid chromatography is a purification technique that makes possible separation of liquid-soluble compounds in either analytical or preparative quantities. Compounds are resolved or separated from each other on the basis of differences in affinity for a solid stationary phase and a liquid mobile phase.

The stationary phase consists of small (5-100 μm in diameter) porous silica particles tightly packed into pressure-resistant steel columns; particle size varies depending on the application. In most reverse-phase columns, only 10-20% of the column volume is occupied by impermeable silica; of the remainder, approximately 35% to 45% is interstitial volume, and 45% to 55% is pore volume (104). Adsorption of solutes to the stationary phase occurs primarily in the pores, so the size of the pores as well as the size of the particles affects retention and elution profiles. Support particles with pore diameters of 50-100 A are best for resolution of small peptides (less

than 30 residues) (105).

The surface character of the silica particles varies also, depending on the application. Reverse-phase (RP) columns are so termed because their normally polar particle surfaces have been end-capped or carbon-loaded with nonpolar hydrocarbon residues. The use of a hydrophobic coating on the silica particles allows use of the hydrophobic effect as a sorptive mechanism. Free silanol groups (R-Si-OH) on the surface of the particles are most commonly bonded to 8- or 18-carbon aliphatic hydrocarbon chains, producing a (usually) monomeric hydrophobic coating over the entire stationary bed surface; cyano and phenyl coatings are also used. Ideal hydrocarbon loading of silica for peptide RP-HPLC applications is roughly 10-12% of the total stationary phase mass. At this carbon load, the surface is almost completely covered with a monomeric C_8 or C_{18} coat; free silanol groups are almost nil. Higher carbon-loading inhibits adsorption of polar solutes, resulting in poor resolution of peptides. Lower carbon-loading has the opposite effect, with basic residues (Lys, Arg) adsorbing too well, yielding broadened peaks or even irreversible binding of solutes to the column (105,106).

The mobile phase used in RP-HPLC consists generally of two or three solvents differing in polarity; the proportions of the solvents are varied over time to effect the most efficient separation of peptides by the column. The mobile phase is passed through the column under high pressure (800 to 2000 psi, roughly, for analytical or semi-preparative columns) in order to produce a relatively quick flow rate through the column bed. The most common solvents used in

RP-HPLC are water and acetonitrile, usually used with ionic mobile phase modifiers or counterions such as trifluoracetic acid (TFA) or hepta-fluorobutyric acid (HFBA).

Separation of the solutes present in a sample is based on the principle that each peptide will have different affinities for the mobile and stationary phases than most of the other peptides in solution. In the simplest form of separation, a single-component mobile phase would wash two different solutes through the stationary phase at different rates because the solutes differed in their adsorption and solubility coefficients. Such a simple system does not provide a high degree of resolution, however: only substances that do not actually bind to the stationary phase (whether by ionic interaction or, as in RP columns, hydrophobic effect) can be eluted, so their interaction with the stationary phase is necessarily weak and elution will occur in a rather broad band. (The tailing effect seen in paper or thin-layer chromatography illustrates this.) Binary mobile phases, consisting of a relatively nonpolar solvent and a relatively polar solvent, can greatly improve resolution of solutes in a reverse-phase application. The solutes are loaded onto the column in a highly polar solvent mix, such that hydrophobic interactions between the C_{18} surface and the solutes are maximized: the solutes bind firmly to the column, and will not elute from the column as long as the polarity of the eluent is maintained. As the mobile phase becomes increasingly hydrophobic, however, the affinity of the solutes for the mobile phase increases. When the solutes' affinity for the stationary phase is equalled by their affinity for

the mobile phase, they begin to dissociate from the C_{18} surface and move with the eluent down the column, re-equilibrating between stationary and mobile phases as they proceed. Each solute elutes from the column at the precise solvent concentration that ideally solubilizes it, in terms of both its hydrophilic and hydrophobic character.

The adsorption of peptides to RP columns is greatly enhanced by the addition of ionic mobile phase modifiers such as TFA to the mobile phase. The mechanism by which interaction with the stationary phase occurs is not dependent upon ion-pair formation in either mobile or stationary phase, nor is it a case of ion exchange. Rather, the mechanism is postulated to involve a dynamic ion-interaction model. This model suggests that the peptide solute molecule interacts with anionic modifier molecules (mobile and stationary), polar and nonpolar mobile phase components, the hydrophobic stationary phase surface, and mobile and stationary simple ions (107, 108, 109). Adsorption and partition both occur by a combination of electrostatic forces and hydrophobic effect. Subtle changes in the composition of the mobile phase will affect retention of solutes; the presence of salt cations in tissue extracts, for instance, will alter the electrostatic forces present and will increase retention times slightly relative to salt-free peptide standards.

Generally speaking, peptides will repeatedly elute from reverse-phase columns at the same concentration of solvent B (the hydrophobic component of the mobile phase)run after run. However, many variables

exist which, if changed, will cause some variation in the retention times and eluting concentration of acetonitrile. These could include: subtle difference in the concentrations of organic solvent (CH_3CN) or ionic modifier (TFA); changes in pitch of the solvent gradient; changing the type of anionic modifier (from TFA to HFBA, for example); or, as discussed above, addition or deletion of cations. More dramatic changes, such as reversal in elution order, can be obtained by more drastic changes in the choice of anionic modifier (e.g. from TFA to undecafluorocaprionic acid) or by changing the type of material used to form the reverse-phase surface (e.g. from a C_{18} coating to $-(CH_2)_3CN$ or $-(CH_2)_2C_6H_6$) (105,108).

In practical terms, resolution of peptides on reverse-phase systems involves choice of appropriate hydrophobic coating, particle size, and mobile phase components, then manipulation of the solvent gradient in order to maximize separation (baseline distance between peaks) or quantitation (amount of peptide in a clearly defined peak). It is often difficult to use the same chromatographic conditions to meet both objectives. For example, two closely related peptides such as somatostatin-28 and dihydrosomatostatin-28 will elute as a single peak on a steep acetonitrile gradient; but by proper manipulation of the gradient, these two entities can be effectively separated to meet either preparative or analytical objectives (see Fig. 5). If a preparative separation is required such that a pure H_2-S28 fraction can be obtained for further experiments, the best method would be to run the gradient up sharply to the eluting concentration of S28 followed by an isocratic period or very shallow gradient to obtain a

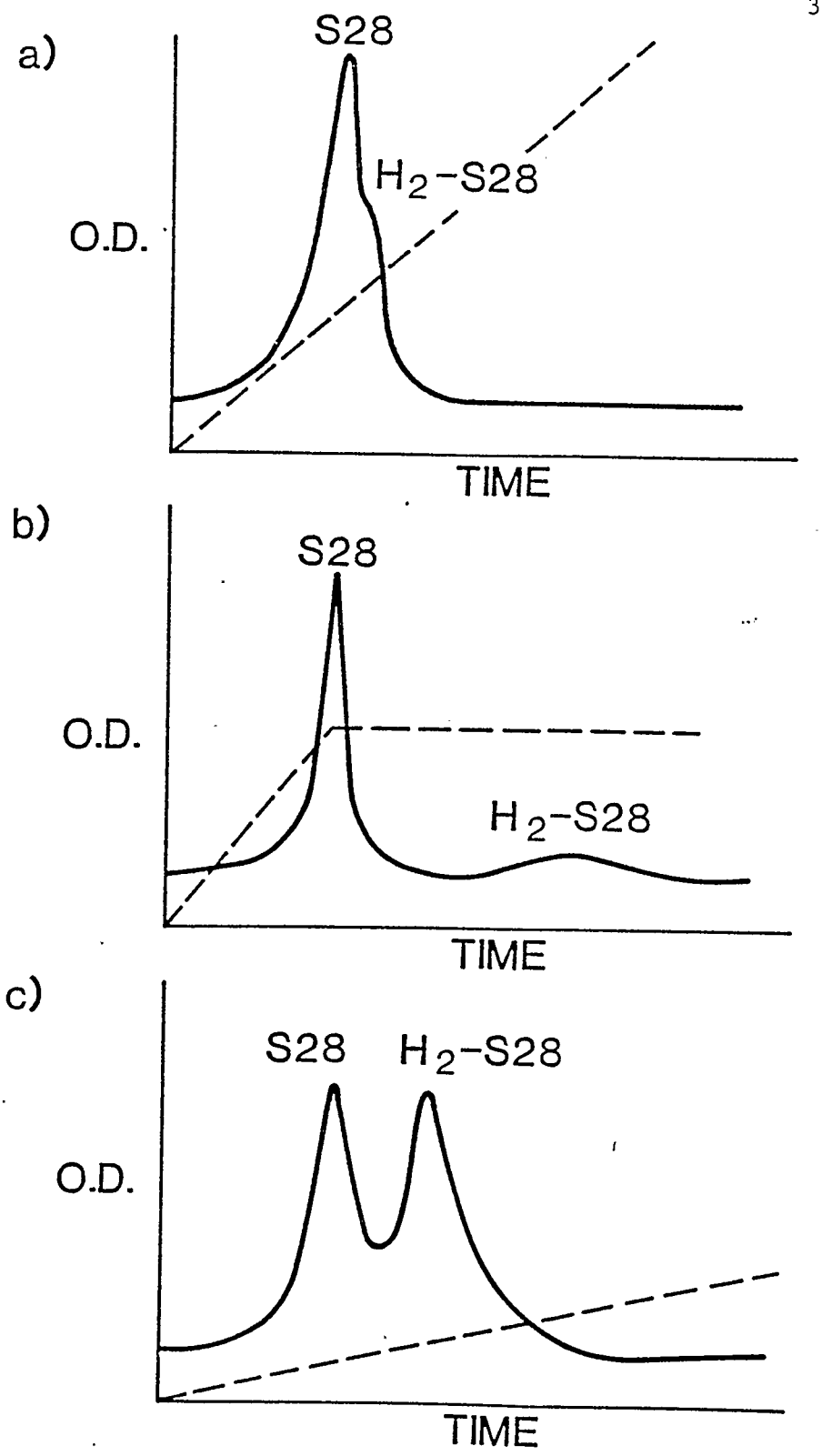

Figure 5: Representative HPLC profiles. Elution of S28 and H_2-S28 under different gradients: a) 3% to 57% acetonitrile over 30 min; b) 3% to 28.5% acetonitrile over 13.5 min, followed by isocratic conditions at 28.5% acetonitrile; c) shallow gradient, 27% to 36% acetonitrile over 30 minutes.

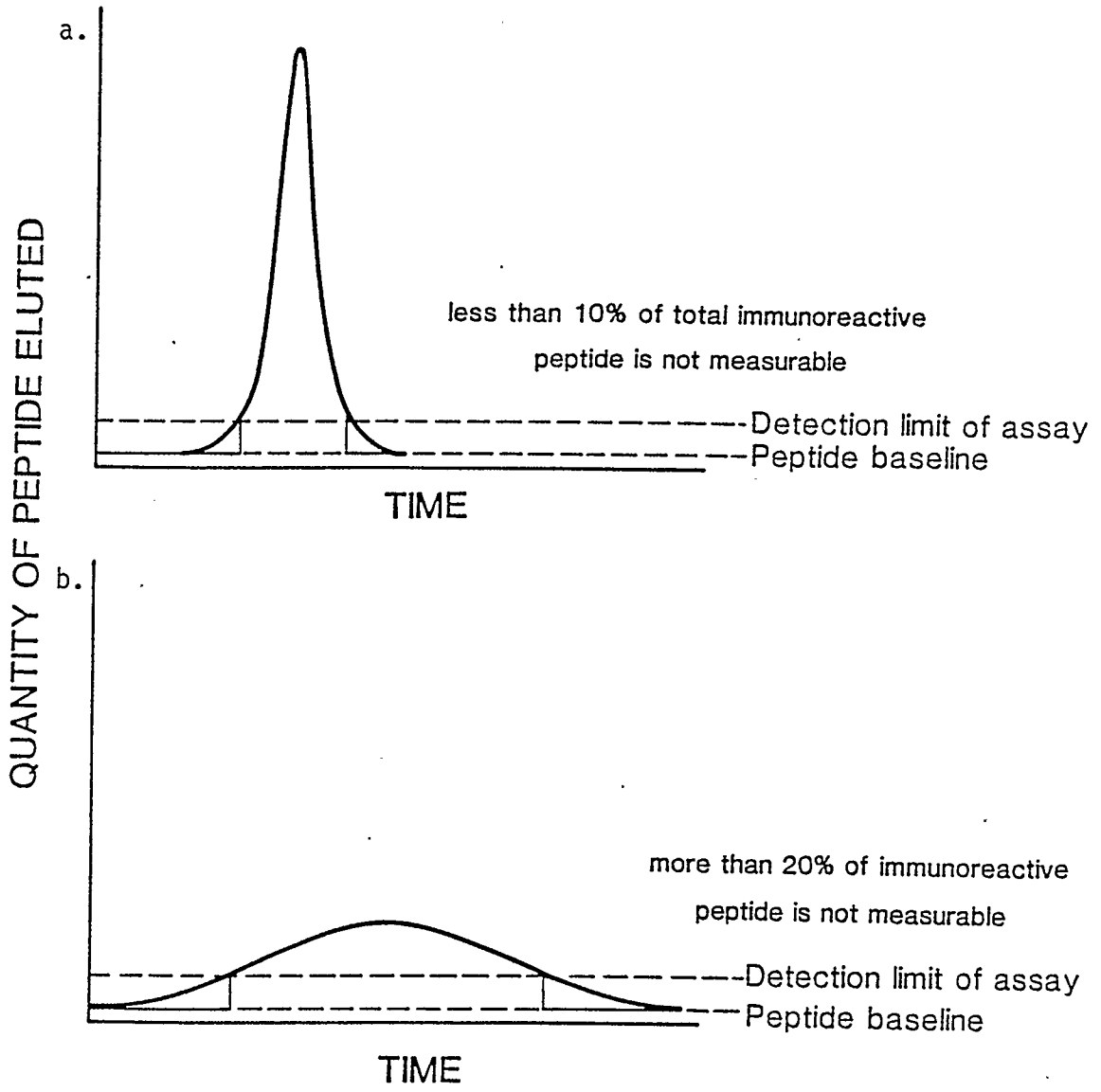

Figure 6: Schematic illustrating relative efficiency of gradient (a) versus isocratic (b) elution of peptide on HPLC.

wide baseline separation between the two peaks. The disadvantage of this method for analytical studies is that the second peak obtained is broad and low. Quantitation of a small amount of the peptide by a separate assay becomes difficult when a broad peak is eluted, because more of the shoulder area of the peak will be below the detection limit of the assay than would be the case with a sharp peak; consequently, the quantity of material apparent will be lower than the actual value (see Fig. 6). For detection of small quantities of a specific peptide, then, a better gradient than either the steep or the steep/isocratic profiles would be a moderately shallow gradient (Fig. 5c) that compromises between the two extremes. This type of gradient may not yield baseline separation between the two peaks, nor will it yield razor-sharp peaks, but it will resolve closely-related substances sufficiently to distinguish them, yet the peaks obtained will not be so broad as to render a significant quantity of peptide below assayable concentrations.

It is apparent, then, that RP-HPLC conditions must be tailored to fit the desired application. Application of the principles of chromatography and familiarity with the practical options available are essential to the development of an optimum chromatographic protocol.

F. Conclusions and Statement of Objectives

Familiarity with the corpus of somatostatin literature inevitably leads to the conclusion that somatostatin in its several forms constitutes a virtually ubiquitous regulatory entity. In addition to its well-known CNS functions, it is an essential component of the

complex regulatory systems that control function of the gastrointestinal system and the endocrine and exocrine pancreas.

Given the broad range of functions performed by somatostatin, it is not surprising that more than one active form of the hormone exists, nor that the major forms (S14 and S28) differ in their apparent potencies in different tissues. The discovery of dihydrosomatostatin in guinea pig brain is a reasonable extension of this trend. Variations in post-translational processing have been clearly established for other prohormones--most notably POMC--in this species, so it is not unreasonable to suggest that three or more products could be synthesized from prosomatostatin.

The evidence for the presence of H_2-S14 in guinea pig brain is sufficient to imply a unique biochemistry in this species with respect to somatostatin that may mean one of two things: that "used" somatostatin is degraded in a markedly different fashion in the guinea pig, or that this animal synthesizes the reduced peptide for a specific biological function.

If dihydrosomatostatin is in fact synthesized to meet a unique physiological objective, several lines of evidence would have to be established to prove the hypothesis true. One of the first of these is to identify tissues containing dihydrosomatostatin and to quantitate concentrations of the peptide therein.

The objective of the research described herein was to address this issue: that is, to attempt to identify and quantify reduced somatostatin in peripheral tissues of the guinea pig; specifically, in pancreas and in selected tissues of the gastrointestinal tract.

II. Materials and Methods

A. Materials

1. Chemicals

Acetonitrile (HPLC Grade) was obtained from Fisher Scientific Co., Calgary. Trifluoracetic acid and hepta-fluorobutyric acid were obtained from the Pierce Chemical Co., Rockford, Illinois.

The following chemicals were obtained from Fisher Scientific Co. and used in the course of extraction, reduction, and radioimmunassay procedures: disodium ethylenediaminetetraacetate (EDTA), sodium chloride, sodium azide, sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$), phenol red, glacial acetic acid, and 5N hydrochloric acid.

Bovine serum albumin, purchased from Sigma Chemical Co., St. Louis, Mo., was used in two grades: RIA grade (Lot #72F-03611), and Fraction V (Lot no.'s 86F-0710 and 86F-0712). Reduction procedures utilized Sigma grade DL-dithiothreitol (DTT, Lot #123F-0143), and Trizma Base, reagent grade (Lot #105F-5637), both obtained from Sigma Chemical Co.

Iodination of tyrosine analogs of somatostatin utilized $Na^{125}I$ from Amersham Co., Arlington Heights, Illinois. Chloramine-T (Lot #A-90) was obtained from Eastman-Kodak Co., Rochester, N.Y. Sephadex G-25 (Lot #13695) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Pierce Protein Assay Reagent (Lot #870501086) was obtained from the Pierce Chemical Co.

Peptides obtained from Peninsula Laboratories Inc., Belmont, CA,

included somatostatin-14 (Lots #011634 and #012274), somatostatin-28 (Lots #006137 and #006743), N-Tyr-S14 (Lot #001179), and N-Tyr-S28 (Lot #005918).

Goat anti-rabbit gamma-globulin (P3 grade, Lot #7TA119) was obtained from Antibodies Inc., of Davis, CA. Normal rabbit serum (Lots #7TA110 and #3ML334) was purchased from the same source. Arnold M4 rabbit somatostatin antiserum was a gift from Dr. M.A.Arnold.

2. Instruments and Apparatus

a) Homogenizers. Preliminary homogenization of tissues was accomplished using a Polytron homogenizer with Kinematica PCU power supply and Polytron 10S generator, obtained from Brinkmann Instruments, Westbury, NY. Fine homogenization was performed with a Thomas 10mL glass homogenizer with Teflon pestle, using a model RZR3 stirrer (Caframo Co., Wheaton, Ont.).

b) Centrifuges. An Eppendorf Microcentrifuge model 5412, used in preparing extracts for HPLC injection, was obtained from Brinkmann Instruments (Eppendorf Gerateban, Hamburg, West Germany). Sedimentation of immune complexes in RIA was accomplished on either an IEC model DPR-6000 centrifuge with IEC model 259 rotor (Damon/IEC Division, Needham Heights, MA) or a Beckmann TJ-6 centrifuge with TH-4 rotor (Beckmann Instruments Inc., Palo Alto, CA).

c) HPLC. The HPLC system used was supplied by Waters Associates of Milford, MA, and consisted of model 510 pumps, model 720 system controller, model 730 data module, WISP 710 autoinjector,model 441 absorbance detector with extended wavelength module, and model U6K

manual injector.

Columns used were Waters Associates Bondapak C18 (3.9mm x 30 cm and 3.9mm x 15cm) and Novapak C18 (3.9mm x 15cm). A Waters LC precolumn filter was also used, self-packed with Bondapak C18/Corasil packing material.

HPLC grade water for chromatography solvents was obtained from a Milli-Q water system (Millipore Corp., Bedford, MA).

d) Fraction Collectors. HPLC fractions were collected on a Gilson model 202 fraction collector, obtained from Gilson France SA, Villiers LeBel, France. Radiolabelling experiments utilized a Gilson MicroFrac-tionator, from the same source.

e) Gamma Counters. Radioactivity of labelled samples was measured on two instruments: a Beckmann Gamma 5500, and an LKB-Wallac model 1274 RIAGamma (Wallac Qy., Turku, Finland).

f) Lyophilizer. HPLC fractions were dried on a Virtis model 10-148 continuous MRBA Lyophilizer, from the Virtis Co., Gardiner, NY.

g) Spectrophotometer. Protein determinations were performed using a Beckmann model DU-8 spectrophotometer.

h) Miscellaneous. A Radiometer model 26 pH meter (Radiometer Copenhagen, Denmark) was used for all pH determinations in buffer preparation. Chemical masses were determined on either a Mettler 1200 N or Mettler AJ100 balance (Mettler Instruments AG, Zurich, Switzerland). Tissues were weighed on a Roller-Smith Precision Balance, from the Biolar Corp., North Grofton, MA. Fixed-temperature incubations were performed in an Isotemp oven, from Fisher

Scientific. Pipettors used included Gilson models P200 and P1000 (Gilson France SA) and an Eppendorf Repeater 4780 (Brinkmann Instruments). RIA tubes were mixed using a Vortex-Genie mixer (Scientific Industries, Inc., Bohemia, NY).

3. Animals

All experiments used male guinea pigs in the 350-400 gram class. Animals were obtained from Charles River Canada, Inc., St. Constant, Que.

B. Methods

1. Extraction of Somatostatin from Tissues

The method used in these experiments to extract somatostatin and related peptides from guinea pig tissues is the standard hot acetic acid extraction (22,29).

Male guinea pigs (350-400g) were sacrificed by decapitation; the body cavity was opened and packed with ice immediately postmortem. The visceral organs of interest were then dissected out: pancreas (whole; n=6), stomach (antrum; n=6), duodenum (first 5 cm immediately distal to pylorus; n=6), midgut or ileum (5 cm section of small intestine beginning at a point 15 cm from pylorus; n=5), and colon (5 cm section of sigmoid colon; n=5). Tissues were cleaned, patted dry, weighed, then immersed in 2-3 mL of cold (4°C) 0.1 mol/L acetic acid. They were then homogenized by three pulses with a Polytron homogenizer on ice and then immersed in a boiling water bath (approximately 96°C) for 10 minutes. After boiling, the tissue homogenates were cooled on ice, then further homogenized using a teflon and glass homogenizer using 8 strokes at 800 rpm. The final

homogenates were then frozen and kept at -25°C for 3-12 hours (30). A small sample of each homogenate (about 150 microlitres) was withdrawn and stored frozen to be used in determination of protein concentrations.

The homogenates were thawed at room temperature, then centrifuged at 2000 x G for 30 minutes. The supernatant was withdrawn and sufficient neat trifluoracetic acid (TFA) was added to each extract to yield a 1% TFA solution (v:v). The acidified extracts were centrifuged in an Eppendorf benchtop microcentrifuge for 15 minutes; supernatants were aspirated and spun again for 15 minutes, the final supernatant then being drawn off and dispensed in 100 microlitre aliquots to be stored at -25°C.

2. Reverse-Phase HPLC

Reverse-phase HPLC of the extracts obtained from guinea pig tissues was performed on a Waters HPLC system as described in section II.A.2.c). Most of the chromatography comprising this report was performed on the μBondapak 30 cm column, owing to its better resolution of S28 and H_2-S28. The system was operated using 0.1% $TFA_{(aq)}$ (solvent A) and 0.1% TFA in 60% acetonitrile/40% water (solvent B) at a flow rate of 1 mL per minute.

Acidified tissue extracts were thawed and spun in an Eppendorf benchtop microcentrifuge to precipitate any insoluble elements still present. The supernatant was injected onto the HPLC column under initial conditions (60% solvent A/40% B). The gradient was then run up to 55% B/45% A over 30 minutes, followed by a column wash: 55% B to 100% B in 1 minute, isocratic at 100% B for 3 minutes, then a

return to initial conditions. Peptide standards were run on the same gradient as the tissue extracts at the beginning and end of each chromatographic experiment. A fast column wash (40% to 100% B in 3 minutes, 100% to 5% B in 3 minutes, 5% to 40% B in three minutes) was interspersed between chromatographic runs to minimize carryover of peptides from one extract to the next. In addition, blank runs followed the column wash after running standards to ensure no carryover occurred, since the concentrations of standards used were many times greater than the concentrations of SLI in the tissue extracts.

Although levels of SLI were well below the detection limit of the UV detector in extract runs, UV absorbance at 214 nm (0.1 AUFS) and backpressure were monitored in order to monitor the gross functioning of the HPLC system. Evidence of unusual UV absorbance or fluctuations in backpressure were considered sufficient grounds for re-chromatographing extracts.

Fractions were collected at 30 s intervals (0.5 mL). The fractions were then frozen at -80°C and lyophilized at room temperature. Dried fractions were stored at -25°C pending resolubilization for radioimmunoassay.

3. Preparation of Radioligand for Radioimmunoassay

Since native S14 and S28 contain no tyrosine or histidine residues suitable for conjugation with radioactive isotopes of iodine, it was necessary to use somatostatin analogs. Several tyrosine-substituted analogs have been used and described in the literature, including [Tyr-1]-S14, N-Tyr-S14, [Tyr-11]-S14, and [Tyr-

8]-S14 (31). The radioligands selected for use in these experiments were ^{125}I-N-Tyr-S14 and ^{125}I-N-Tyr-S28. N-terminally modified analogs have the advantage over centrally-modified analogs in that the former do not alter the conformation of those residues responsible for the conformation of the molecule, nor do they interfere with antibody binding to the central region (31). Also, because the central region of the molecule is not altered, antisera directed to the central region can be used in RIA; this has the advantage of allowing detection and quantitation of N-terminally extended forms of somatostatin such as S28, S25, S20, and proS.

Iodination was accomplished according to the method of Patel and Reichlin (21). The tyrosine analog (5 micrograms) was mixed with 25 μL of 0.5 mol/L phosphate buffer (pH 7.4) and 25 μL of a 0.15 mg/mL chloramine-T solution. To this was added 10 μL of Na^{125}I (1 mCi). The reagents were slightly agitated for 1 minute, following which the reaction was quenched by the addition of 0.5 mL of a 10% BSA solution (RIA Grade). Since chloramine-T iodinates by an oxidative mechanism, it is usually terminated with a reducing agent such as sodium metabisulfite. However, the disulfide bond of somatostatin can be disrupted by reducing agents, so BSA is used to interrupt the reaction without compromising the tertiary structure of the peptide (35).

The reaction solution was then fractionated on a Sephadex G-25 column (1 x 50 cm) eluted with a 0.1 mol/L acetic acid/0.1% BSA running buffer. Passage of the iodination mixture through the gel column yielded three distinct peaks of radioactivity corresponding to

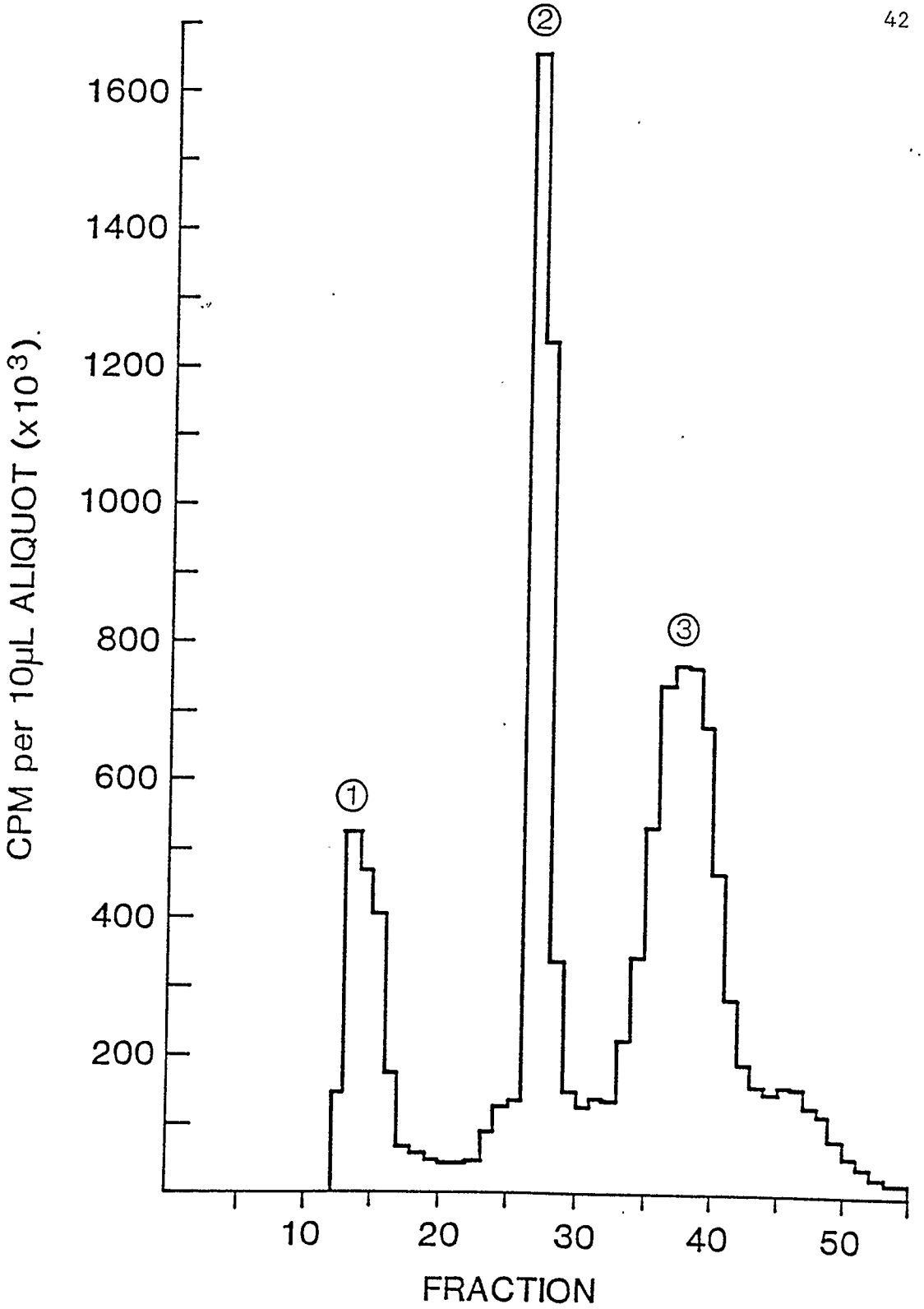

Figure 7: Iodination chromatographic profile: radioactivity of fractions eluted from a Sephadex G25 column (1 x 50 cm) using 0.1 mol/L acetic acid/0.1% BSA eluent. Peak 1 represents ^{125}I bound to BSA; peak 2 represents free ^{125}I ion; peak 3 is the iodination product. Non-labelled somatostatin elutes later than the labelled peptide.

iodinated BSA (void volume), unincorporated iodide, and the radioligand, in that order (see Figure 7). Radiolabelled peptide separates from non-labelled peptide on the G-25 column; the cold peptide elutes later than the iodination product (21). Fractions of approximately 1.5 mL were collected and assayed for radioactivity; the two or three highest fractions of the third peak were pooled for use in RIA.

4. Radioimmunoassay

a) Somatostatin Antiserum

All radioimmunoassays used a centrally-directed rabbit antiserum raised against synthetic S14 by Dr. M.A.Arnold (batch M4) (8). This antibody exhibits a high titer (1:100 000) and, because it recognizes the central portion of somatostatin, it is capable of detecting S28 on an equimolar basis; N-terminally extended forms of the peptide are less well-bound (31). It is established that H_2-S14 spontaneously oxidizes under RIA conditions, and exhibits a parallel competitive binding curve to the standard, S14 (31, see also Results).

b) Assay Conditions

Radioimmunoassay of HPLC fractions was accomplished using a standardized RIA buffer consisting of EDTA (0.01 mol/L), sodium chloride (0.05 mol/L), bovine serum albumin (RIA grade, 0.1%), and sodium azide (0.02%) in 0.1 mol/L sodium phosphate buffer (pH 7.4). (Phosphate buffer was prepared according to the method of Gomori [111].) Solutions of monobasic and dibasic sodium hydrogen phosphate were prepared [0.5 mol/L], then mixed in the proportion 70 mL

monobasic to 430 mL dibasic; pH was adjusted to 7.4 by dropwise addition of the appropriate buffer.) A trace of phenol red indicator was added to the buffer to allow the pH of the assay tubes to be visually monitored. The buffer was prepared in bulk on a regular basis and stored at 4°C.

Sodium chloride was included in the buffer to increase the tonicity of the solution and thereby avoid denaturation of antiserum proteins at extreme dilutions; BSA helped in this regard, but has the additional effect of minimizing nonspecific adsorption of antibody and radioligand to the glass vessels used. EDTA was included to chelate divalent cations, since these species are known to interfere with some antibody-antigen interactions. Azide was added to discourage bacterial growth in the buffer, which was often stored for more than a week prior to use (36).

RIA's were prepared on ice (4°C) and maintained at this temperature for the duration of incubation and centrifugation; the M4 antiserum performed best at cold temperatures. All components of the RIA reaction mixture were prepared and and stored at 4°C.

Dried HPLC fractions were solubilized in RIA buffer at a dilution factor of 2:1 (relative to fraction volume prior to lyophilization) for duodenum, midgut, and colon extracts; pancreas and stomach extracts were solubilized in a 4:1 dilution. Serial dilutions were then prepared for each fraction: 4:1 and 8:1 for intestinal extracts, and 8:1, 16:1, and 32:1 for pancreas and stomach.

Aliquots of 100 microlitres were taken from these dilutions and

added to 300 μL of RIA buffer in 12 x 75 mm glass tubes. Radioligand was diluted in RIA buffer to yield a final concentration of approximately 10^{-8}mol/L; 50 μL of this solution was then added to the assay tubes. Last, 50 μL of 1:10 000 dilution Arnold M4 antiserum was added to yield a final assay volume of 500 μL. The assay tubes were then vortexed and incubated for 24 h at 4°C prior to addition of precipitating antibody.

Separation of free and bound antigen was accomplished by the second antibody technique. Goat anti-rabbit gamma globulin was prepared at a dilution of 1:17.9 in RIA buffer; normal rabbit serum at 1:71.4 dilution was also prepared; addition of 100 μL each of GARGG and NRS to the assay tubes yielded final dilutions of 1:125 and 1:500, respectively. Assay tubes were agitated again and incubated for a further 24 h.

This second incubation was then terminated by centrifugation at 2000 x G for 30 minutes. The supernatant, containing free radioligand, was aspirated, and the radioactivity of the pellets was determined using 1 minute counting periods on a gamma-counter.

c) Assay Controls

Each RIA was preceded and followed by duplicate control tubes to determine total antigen radioactivity included in the assay, nonspecific binding of radioligand to assay tubes, and reference binding of radioligand to antibody in the absence of nonradioactive antigen. Nonspecific and reference binding were assayed in a total volume of 0.5 mL and were incubated and precipitated in the same fashion as the assay.

In addition, a standard antigen inhibition curve was generated for each assay by preparing serial dilutions of standard S14 (1.95, 3.9, 7.8, 15.6, 31.2, 62.5, 125, and 250 fmol per 100 μL) and adding these to assay tubes in the place of diluted HPLC fractions.

Quantitation of SLI in each fraction was accomplished by subtracting non-specific binding from each tube's radioactive count and calculating bound radioactive antigen (B) as a percentage of reference binding (B_0). This value was then located on the standard displacement curve for S14 and the estimated quantity of SLI was read from the corresponding axis. At least 2 dilutions of each SLI-containing fraction were assayed, and average SLI values were calculated therefrom.

5. Protein Assay

Total protein concentrations of tissue homogenates were determined by the Bradford protein assay (110) using a commercially available reagent. The Bradford assay was selected over the Lowry assay because of its speed, simplicity, and superiority with respect to interfering agents such as potassium and magnesium ions and DTT. The latter agent was originally considered as a stabilizing agent for reduced peptides in the extraction process, so for the sake of consistency it was decided at the outset to use the protein assay for all homogenates that would not be adversely affected by this reducing agent.

Homogenates were prepared for assay by diluting to twice initial volume in 0.1 mol/L acetic acid. Bovine serum albumin standards were prepared containing 5, 10, 15, 20, 25, 50, 75, and 100 μg BSA, and

all standards, diluted homogenates, and blanks were topped up to an equal volume of 1 mL. To each tube 1 mL of protein assay reagent was added. The tubes were mixed thoroughly and read immediately at 595 nm on a Beckmann spectrophoto-meter. Two readings were obtained for each sample and standard, and mean values were calculated. Estimations of protein concentrations of samples were obtained by constructing a BSA standard curve and extrapolating protein concentrations from the mean A_{595} for each homogenate dilution.

6. Reduction of S14 and S28

Reduced forms of S14 and S28 were prepared in the laboratory for use as elution determination markers for HPLC and as standards in RIA control experiments.

H_2-S14 and H_2-S28 were prepared from commercial S14 and S28 by reacting 5 μg of peptide (equivalent to 3.1 nmol S14 or 1.4 nmol S28) with dithiothreitol. The peptides were dissolved in 220 μL of DTT solution (5mmol/L DTT in 0.3 mol/L Tris-HCl buffer, pH 8.1). The reaction tubes were flushed with nitrogen, capped, and incubated at 50°C for 1 hour, after which they were cooled on ice (29, 30).

Separation of the reduced peptides from DTT was accomplished using HPLC under the conditions described previously; DTT elutes in the void volume. Peptide fractions were collected, lyophilized to remove acetonitrile and TFA, then resuspended in 0.1 mol/L acetic acid and stored at -80°C in 1 μg/50 μL aliquots.

III. Results

The experiments performed to determine the nature and quantities of somatostatin-like immunoreactivity in the guinea pig showed that SLI in the guinea pig's peripheral tissues is in many respects similar to that in the rat and other mammals, but in other ways it was quite different. The same major forms of somatostatin (S28 and S14) were found in all tissues, as expected, although the proportions of these did not necessarily follow typical mammalian patterns. In addition, dihydrosomatostatin-14 was identified in all the tissues examined; H_2-S14 has previously been reported in guinea pig brain structures, but in no other species.

A new finding of these experiments was the identification of dihydrosomatostatin-28, again in all tissues examined.

A. Recovery of Peptide from Tissue

Tissues from pancreas, antrum of stomach, and duodenum (n=6), and from midgut (ileum) and sigmoid colon (n=5) were subjected to hot acetic acid extraction as previously described. Control experiments were performed by adding exogenous radiolabelled somatostatin-14 and somatostatin-28 analogs to the tissues prior to extraction. Results indicated a mean recovery of 33% of radiolabelled peptide after extraction (23% of ^{125}I-N-Tyr-S14, 42% of ^{125}I-N-Tyr-S28).

The bulk of peptide loss appeared to be due to non-specific binding to insoluble elements of the homogenate: 55% of the radiolabel was lost in the first two centrifugations.

Total recovery of peptide after extraction and chromatography was low (24.4%), although this figure is not inconsistent with

recoveries reported in the literature (22,103).

B. HPLC of Peptide Standards and Tissue Extracts

High performance liquid chromatography was performed as described previously. The indicated gradient on this HPLC system enabled separation of four standard peptides: S28, H_2-S28, S14, and H_2-S14 (see Fig. 9). These peptides eluted over a period of 7-8 minutes, with no less than one minute separating any two peaks. Peak broadening and the consequent problem of carryover into several subsequent fractions was kept to a minimum by maintaining an optimal gradient. Resolution of the two smaller forms, S14 and H_2-S14, was very clean: near-baseline separation was achieved, with approximately 1.5 minutes between peaks. Baseline separation of the smaller pair (peaks 3 and 4, Fig. 9) from the larger pair, S28 and H_2-S28 (peaks 1 and 2), was not difficult. Resolution of S28 from H_2-S28 was less successful: S28 tended to elute as a broader peak than S14 under these chromatographic conditions, yet a steeper gradient failed to resolve the two 28-residue forms; consequently, these two eluted as a doublet.

Chromatography of standard somatostatin-20, a degradative fragment of S28, produced a peak that co-eluted with S28 and approximately one minute earlier than elution of standard dihydrosomatostatin-28, ruling out the possibility that the second peak of the doublet was S20.

Chromatography of tissue extracts yielded profiles with measurable SLI at retention times other than those of the four

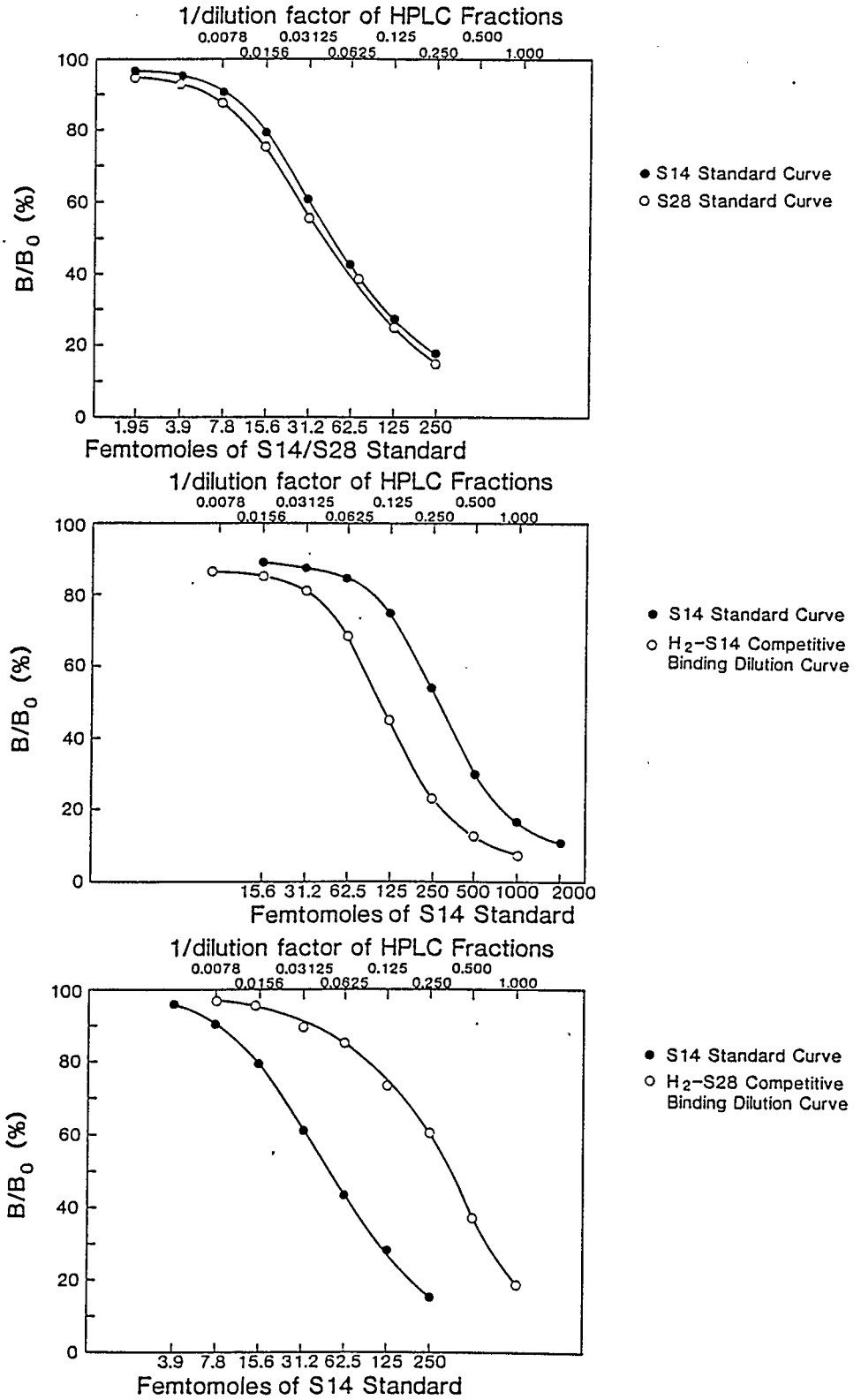

Figure 8: Comparison of S28, H_2-S14, and H_2-S28 competitive binding curves with S14 standard binding curve.

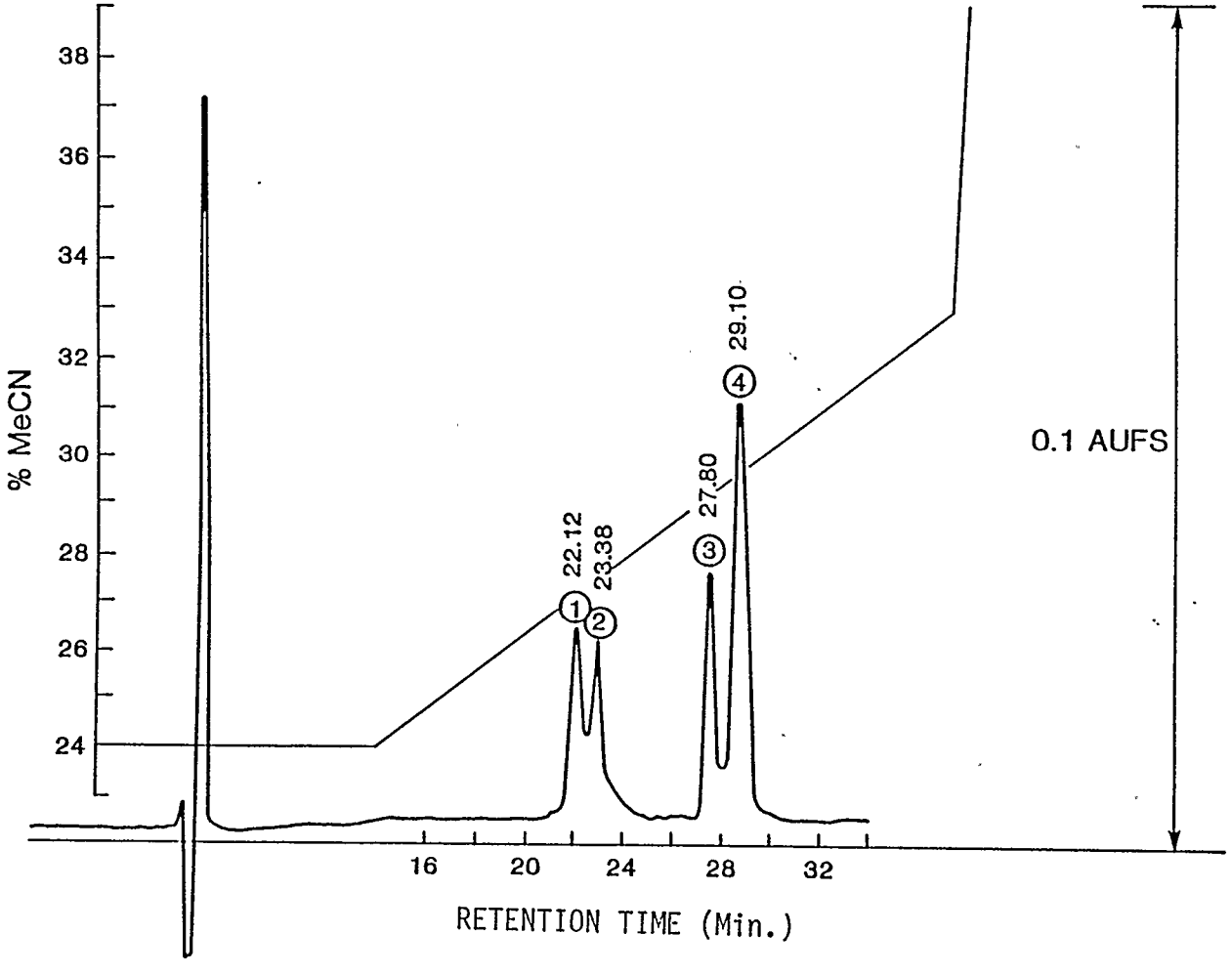

Figure 9: High performance liquid chromatogram of elution of somatostatin standards (approx. 1.0 g each) from a reverse-phase column using a 27% to 36% acetonitrile gradient over 30 minutes. Indicated peaks and CH_3CN eluting concentrations are: 1) S28 (27.0%), 2) H_2-S28 (27.5%), 3) S14 (29.0%), 4) H_2-S14 (29.8%).

standards used (see Fig. 11), notably prior to elution of S28, between the S28/H_2-S28 doublet and the S14/H_2-S14 pair, and later than H_2-S14. Early and intermediate unidentified SLI most likely represented degradative fragments of somatostatin that, although partly degraded, remained intact in the central region of the molecule recognized by the RIA. Late SLI was presumed to be precursors to the 28- and 14-residue biologically active forms.

C. RIA Quality Control

Radioimmunoassay of somatostatin standards and of fractions obtained by HPLC was performed with acceptable reproducibility throughout the research project. Quality control records were kept for all assays: average non-specific binding (NSB) of radioligand was 5.3%, average reference binding (B_0) was 42.2%, and average median displacement (50% B/B_0) was 25.1 fmol over the course of 108 RIA's.

Because the antibody used recognizes the central portion of S14, a region very close to the C-terminus of any N-terminally extended forms of the peptide, all molecular forms of somatostatin and its precursors were detectable by RIA. Figure 8 illustrates comparison of competitive binding dilution curves for S28, H_2-S28, and H_2-S14 with the S14 standard dilution curve. S28 and H_2-S14 exhibited close to parallel curves, suggesting that they compete equipotently with S14 for antibody binding sites. H_2-S28 produced a less perfect parallel curve; it is possible this form does not compete for binding sites on an equal basis with the other three molecular forms. Larger

well than the major forms (29, 31).

D. Total Somatostatin-Like Immunoreactivity in Extracts

Total somatostatin-like immunoreactivity for the extracts of the five tissues studied is tabulated in TableI and illustrated in Figure 10. Highest immunoreactivity was seen in pancreatic extracts, both as a function of SLI per unit wet weight and as SLI per unit of soluble protein. Stomach extracts held significantly lower levels of SLI per milligram wet weight; but because of the presence of dense muscle in gastric tissue, comparison with pancreatic concentrations is likely more valid when SLI per milligram protein values are compared. On these terms, total stomach SLI is only slightly less than pancreatic concentrations, and significantly higher than any gut tissue extract.

Of the intestinal regions studies, lowest immunoreactivity was detected in the ileum. Duodenal extracts displayed highest immunoreactivity per unit wet weight, probably indicating a high density of somatostatin-secreting cells and a concomitant low muscle mass. Sigmoid colon, although containing low SLI concentrations per mg wet weight, surprisingly had the highest concentration of SLI per unit protein of the three intestinal regions examined.

E. Chromatographically Separable Forms of SLI

1. HPLC Profiles of Tissue Extracts

Immunoreactivity profiles of chromatographed tissue extracts are illustrated in Figure 11. Generally speaking, the

Tissue	Somatostatin-like Immunoreactivity	
	pmol/mg wet wt.	pmol/mg protein
Pancreas	0.740 ± 0.290	82.5 ± 17.4
Stomach	0.368 ± 0.120	74.7 ± 16.6
Duodenum	0.276 ± 0.0950	27.4 ± 7.50
Midgut	0.143 ± 0.022	16.2 ± 4.10
Colon	0.221 ± 0.079	37.2 ± 17.0

Table I: Total somatostatin-like immunoreactivity in five peripheral tissues (mean ± S.E.M.)

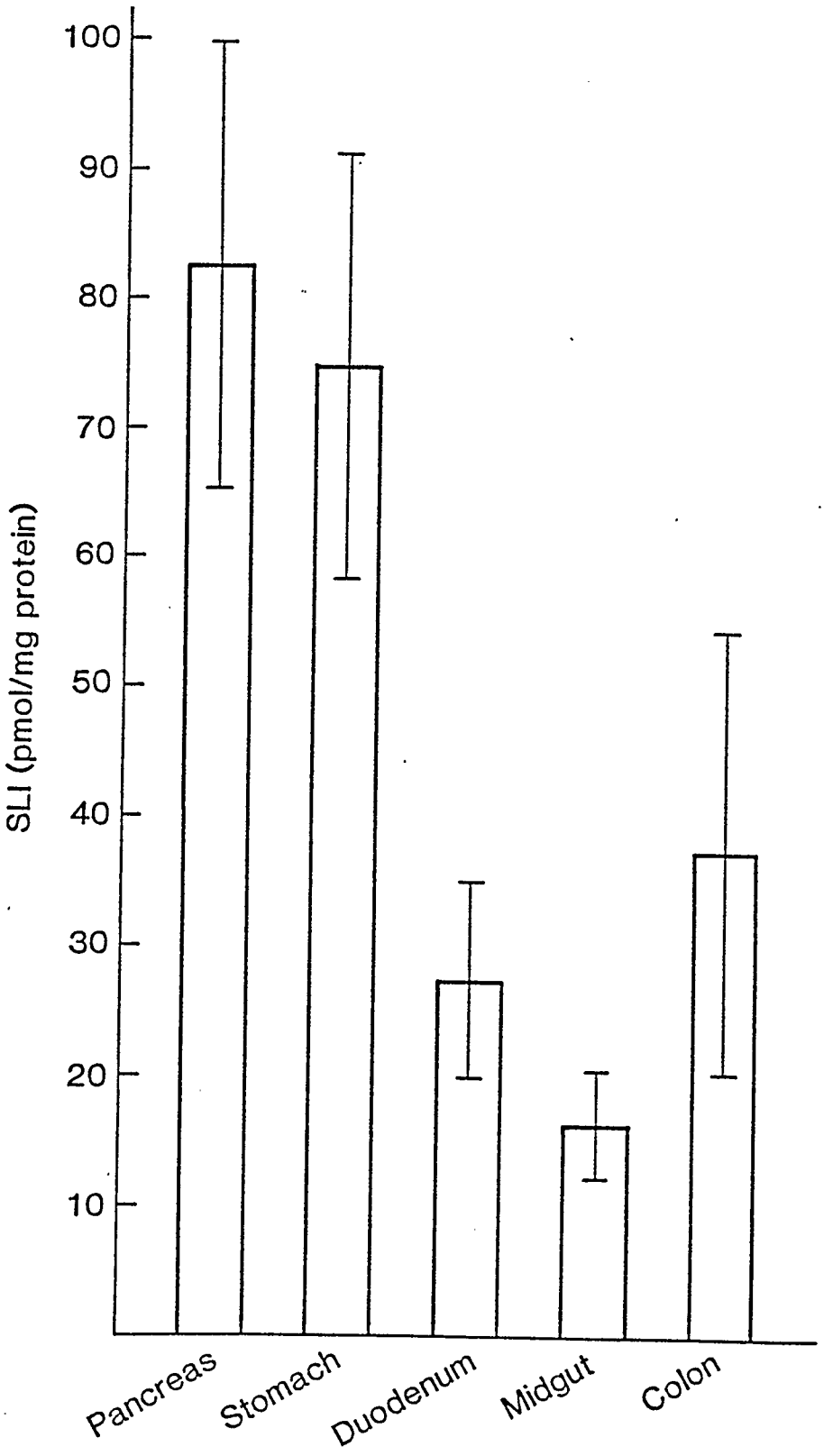

Figure 10: Total somatostatin-like immunoreactivity (SLI; picomoles per milligram protein, ± S.E.M.) in each tissue assayed.

profiles obtained in these experiments exhibited much lower late SLI (corresponding to the pro-hormone) than did brain extracts from the guinea pig (see Ref. 29).

Pancreatic extract profiles exhibited very high consistency from animal to animal. They did not strictly conform to the pancreatic pattern seen in most mammals: clearly discernible peaks corresponding to S28, dihydrosomatostatin-28, and dihydrosomatostatin-14 were evident. Somatostatin-14 represented the largest component of total SLI. Late SLI was observed in small quantities.

Chromatography of stomach extracts yielded a profile quite consistent with the mammalian gastrointestinal pattern, but only if S28 and H_2-S28 peaks are considered together for purposes of percentage comparisons (likewise for S14 and H_2-S14). Typically, guinea pig stomach extracts exhibited profiles very similar to pancreas, with clearly discernible peaks representing S28 and the dihydro forms of both major peptides. Again, late SLI contributed only a small percentage of total SLI.

The duodenum expressed the greatest variability from animal to animal of all tissues studied, possibly because of differences in feeding times and consequent differences in somatostatin levels in response to lumen contents. S28 was present in highest concentrations, followed by S14. Dihydrosomatostatin-28 and -14 were both present, although in lower concentrations than in any of the other tissues studied. As the HPLC profile shows, duodenal extracts exhibited a great deal of tailing between peaks; baseline separation

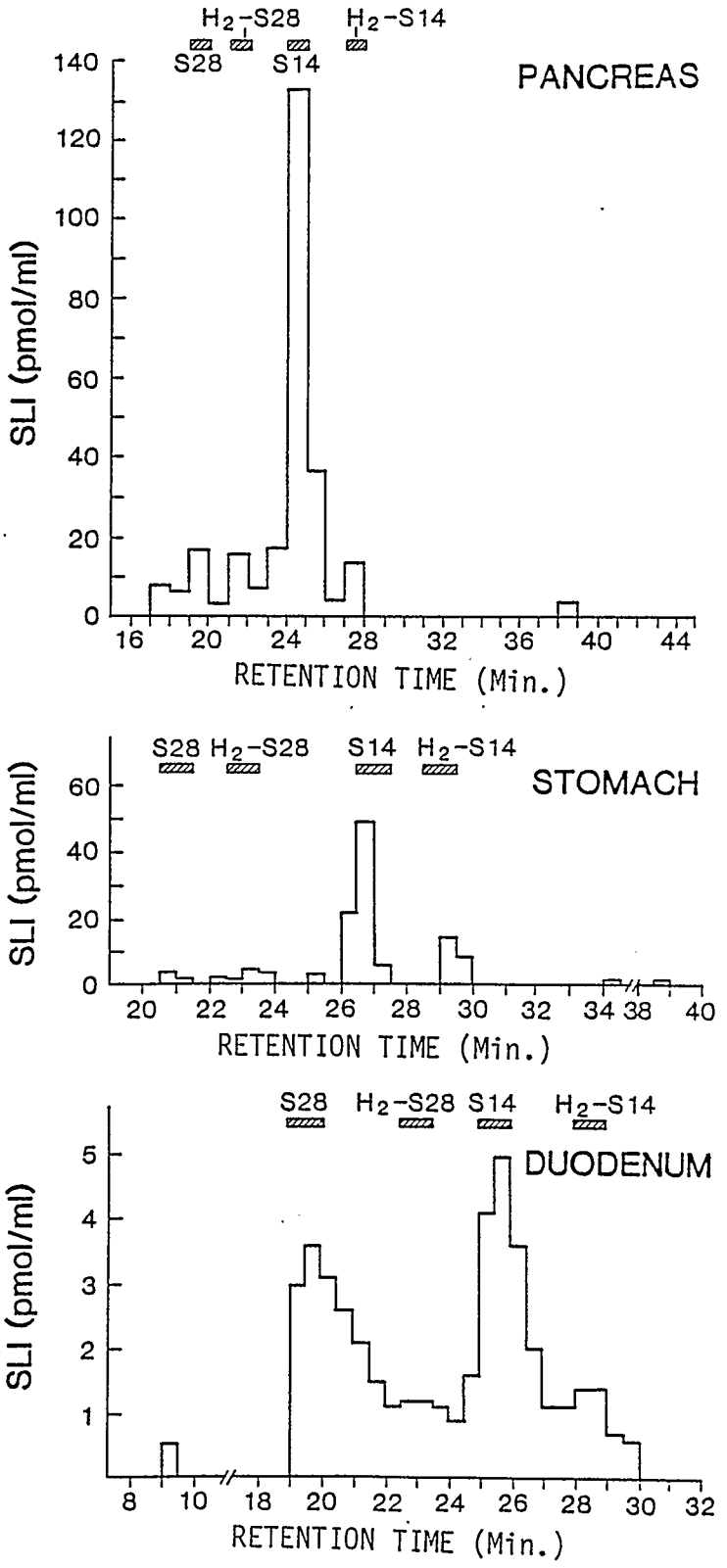

Figure 11: Representative HPLC profiles for each of the tissues studied: somatostatin-like immunoreactivity (SLI) is plotted against retention time. Cross-hatched bars indicate retention times of peptide standards.

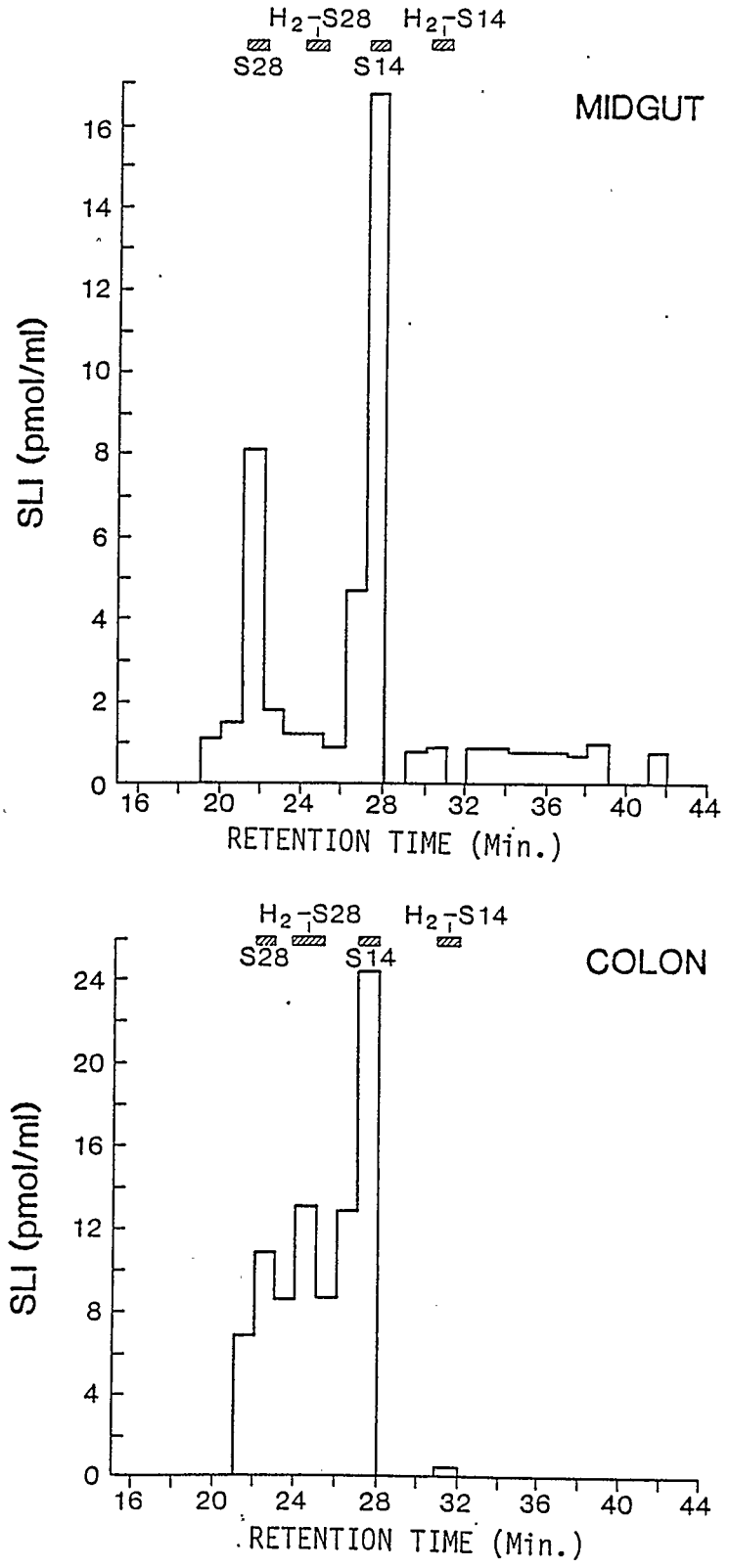

Figure 11 (continued): Representative HPLC profiles for each of the tissues studied.

extract, possibly due to high levels of immunoreactive degradative fragments in this tissue. Nonidentifiable SLI therefore constituted a mean 16% of total SLI. Again, the results obtained did not conform to the typical mammalian gastrointestinal pattern of SLI.

Midgut (ileum) SLI profiles also deviated from the expected pattern. Somatostatin-14 constituted the major species, followed by S28. Dihydrosomatostatin-28 was quite high by comparison with other tissue, but dihydrosomatostatin-14 was present in very low concentrations. Interestingly, this tissue exhibited the highest late SLI concentration of all 5 tissues.

Chromatography of colonic extracts yielded several interesting results. First, contrary to expectations from studies of SLI in rat (17), SLI per milligram protein was highest in this of the three intestinal tissues. Second, H_2-S28 made up a higher portion of total SLI than in any other tissue. Third, H-S14 was found in only very low concentrations in three animals, and not at all in two animals. Late SLI and unidentifiable immunoreactive fragments were also present in very low concentrations in this tissue.

2. Quantitation of Chromatographically Separable Forms of SLI

Quantitative analysis of SLI according to chromatographically separable molecular forms in each tissue is tabulated in Table II and illustrated in figure 12.

In all tissue extracts except duodenum, S14 was the predominant molecular form; in duodenum S28 levels were slightly higher than S14,

Tissue	S28	H_2-S28	S14	H_2-S14	Other
Pancreas	6.01±0.7	5.63±0.8	6.01±1.5	11.1±2.8	7.16±2.7
	7%	6%	67%	12%	8%
Stomach	6.21±2.2	7.03±2.0	51.1±14	7.37±2.2	4.58±2.1
	8%	9%	67%	10%	6%
Duodenum	9.66±4.7	0.94±.41	9.06±2.4	1.53±0.6	2.52±1.4
	41%	4%	38%	6%	11%
Midgut	5.52±1.0	2.35±.32	7.14±2.3	0.40±0.2	1.69±0.7
	32%	14%	42%	2%	10%
Colon	10.8±3.8	7.4±7.4	29.0±13	2.23±2.2	1.22±0.8
	21%	15%	57%	4%	2%

Table II: Chromatographically separated forms of somatostatin-like immunoreactivity for five peripheral tissues. (Absolute SLI = pmol/mg protein ± S.E.M.)

although the tetradecapeptide's percentage contribution to total SLI was still quite high. Somatostatin-28 was found in low concentrations in pancreas and stomach, accounting for only 7% and 8%, respectively, of total SLI in these tissues; even H_2-S14 and H_2-S28 were more abundant here. In gut tissues, however, S28 was present in much greater quantities. As expected, its contribution to total SLI progressively diminished along the length of the gut (see Figures 13 and 14).

Dihydrosomatostatin-14, already described in guinea pig brain tissues, was present in all five peripheral tissues studied, most notably in pancreas and stomach. It was least abundant in midgut, accounting for only 2% of total SLI in this region, and only slightly higher in colon (4%) and duodenum (6%).

Dihydrosomatostatin-28, a molecular form not previously reported in the literature, was also detected in midgut and colon. Unlike S28, which exhibited a diminishing profile from duodenum to midgut to colon, H_2-S28 increased distally (Figures 13 and 14).

Other forms of somatostatin-like immunoreactivity were found in all tissues, as the HPLC profiles (Fig.11) indicate. These generally made up between 6% and 10% of total SLI in each tissue except in colon, where immunoreactivity aside from the four peptides of interest was almost negligible.

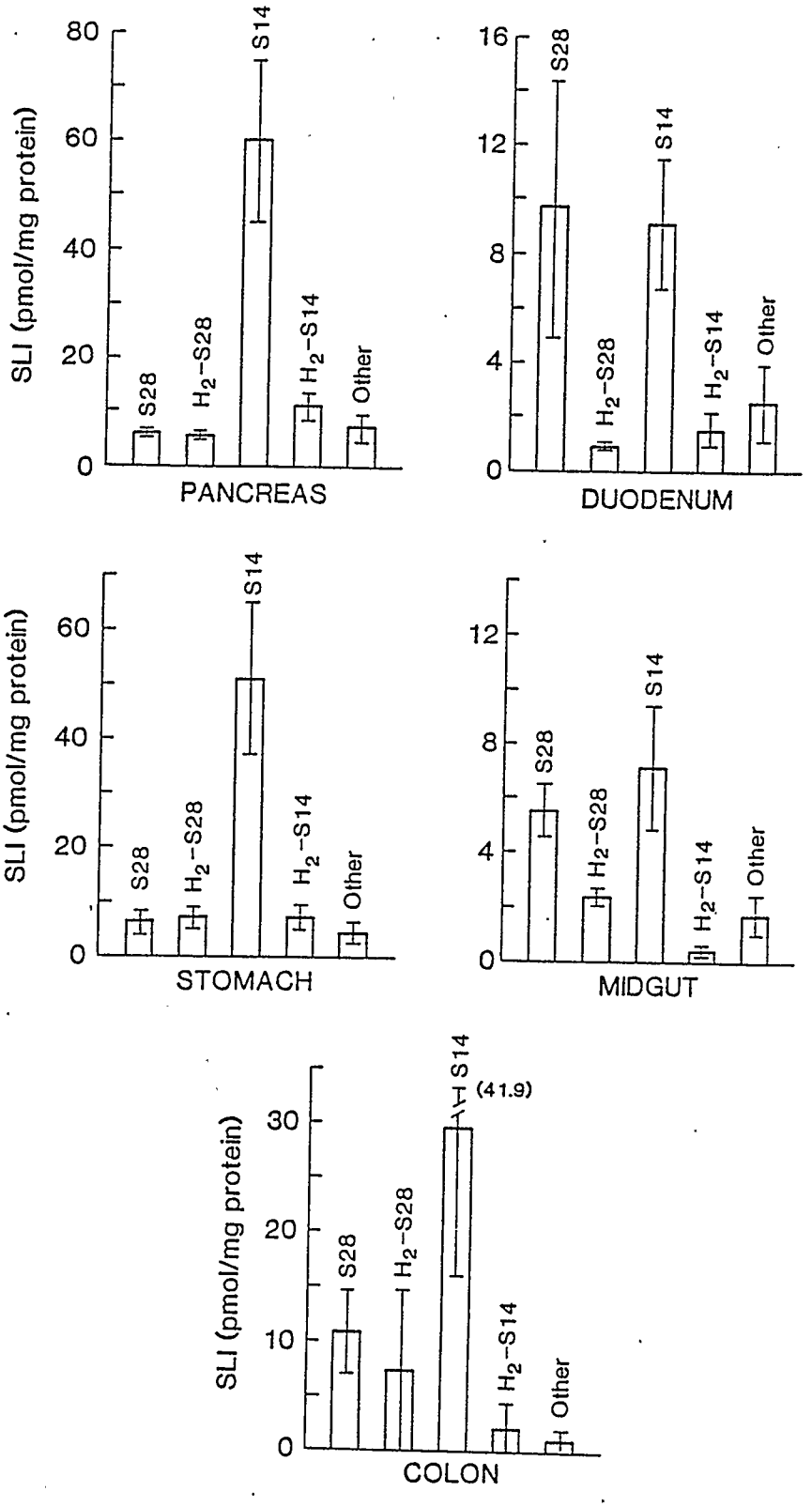

Figure 12: Chromatographically separated forms of SLI in each of the tissues studied(SLI = pmol/mg protein ± S.E.M.)

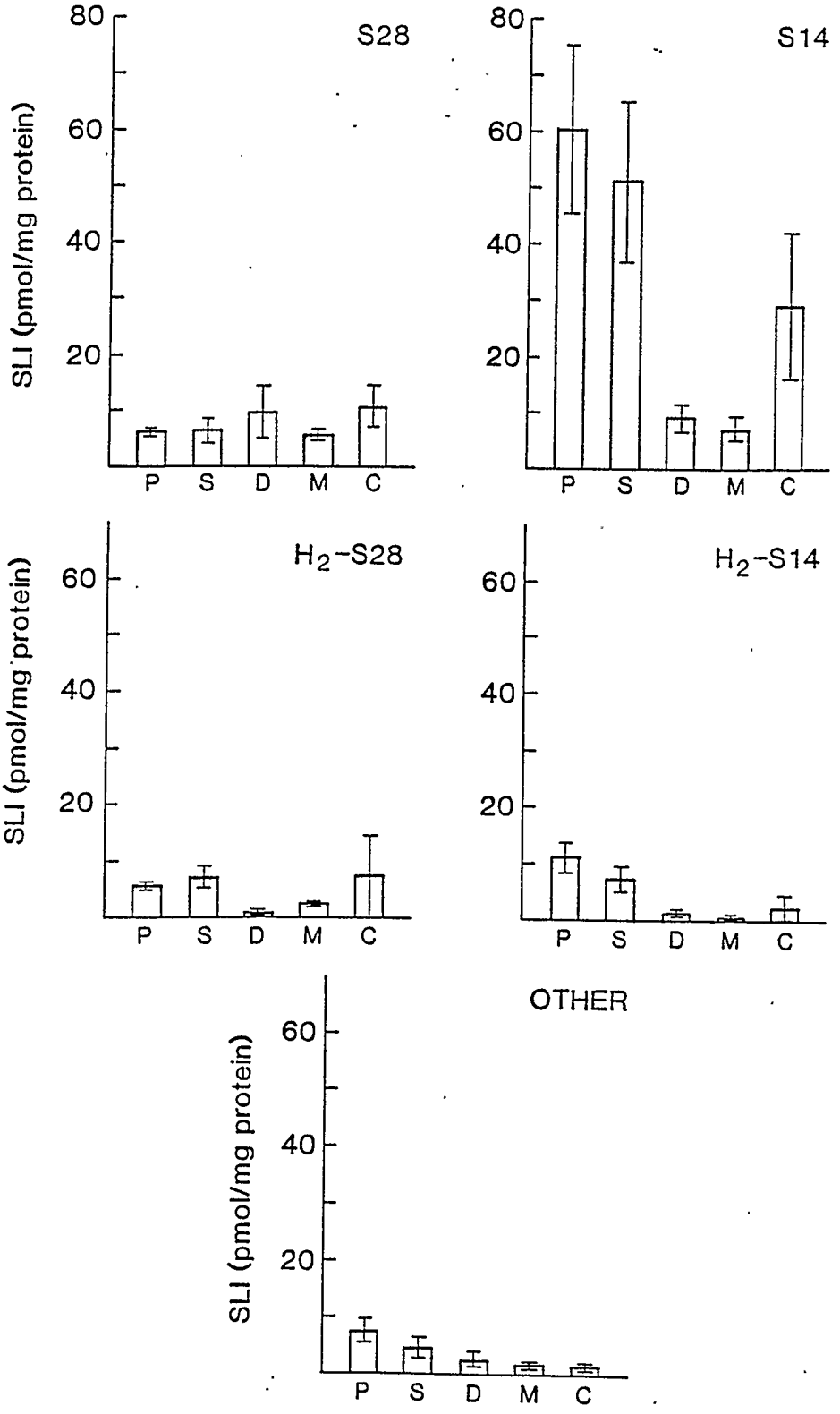

Figure 13: Comparison of molecular forms of somatostatin-like immunoreactivity (SLI; pmol/mg protein ± S.E.M.) in the five tissues assayed (P, pancreas; S, stomach; D, duodenum; M, midgut; C, colon).

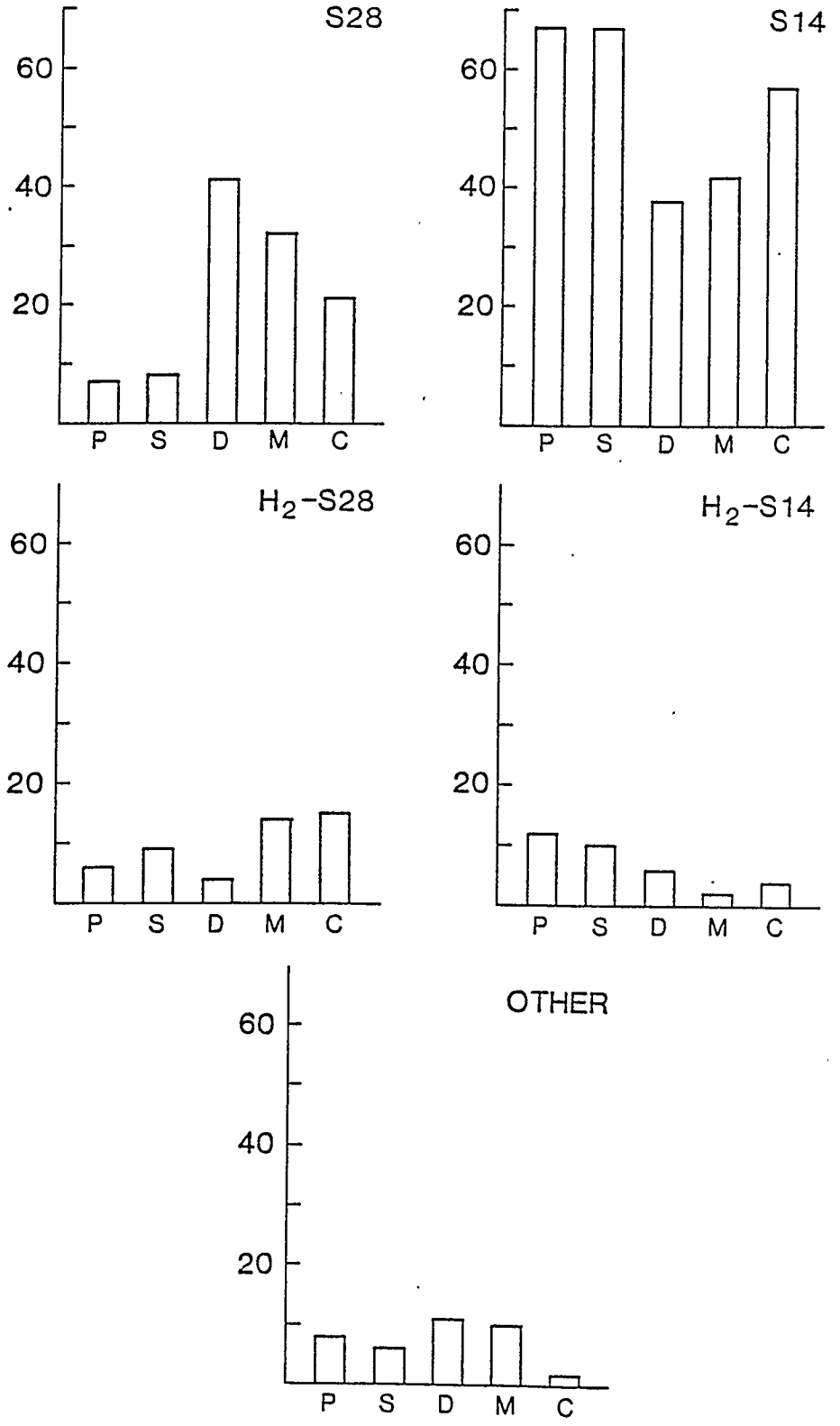

Figure 14: Comparison of molecular forms of somatostatin-like immunoreactivity (SLI; expressed as a percentage of total SLI) for each of the five tissues assayed (P, pancreas; S, stomach; D, duodenum; M, midgut; C, colon).

IV. Discussion

As was noted in the Results, recoveries of SLI from guinea pig tissues were comparable with quantities reported in the literature (22, 29). However, previously published results by other laboratories have not shown evidence for peptides other than S14, S28, and prosomatostatin, although one author has noted identification of multiple molecular forms of SLI in guinea pig intestinal extracts (22). The identification and quantitation of H_2-S14 in guinea pig tissues has been accomplished with brain structures (29), but the results reported in the present study with respect to H_2-S28 are a new finding.

There are two possible explanations for the fact that reduced forms of S14 and S28 have not been identified in previous investigations of guinea pig GI and pancreatic tissues. First, the chromatographic technique used in the present study were more sensitive than those of some previous works: in these studies, extracts were subjected to either ion exchange or gel filtration columns followed by RIA, or large volumes of extract were run on reverse-phase HPLC columns to yield a detectable UV peak of somatostatinlike immunoreactivity (17,22).

A second factor contributing to the suggestions of the present study is that the Arnold M4 antiserum used in radioimmunoassays is capable of reliable and precise detection of peptides in down to concentrations of 5-10 pmol/L. This sensitivity enabled detection of quite small quantities of peptides, which in turn allowed HPLC separation of extracts on analytical columns--as opposed to

semipreparative columns, as would be required for larger quantities of peptides. Use of smaller quantities of extracts in the chromatography portion of these experiments had the added bonus of lengthening the lifespan of both guard and analytical columns and maintaining a high plate count for the full series of runs. Owing to these considerations it was possible to reliably separate and quantitate oxidized and reduced forms of S28 and S14 in guinea pig tissue extracts.

Of course, a third factor contributing to the new findings reported herein is that it is quite possible that dihydrosomatostatin is not present in tissues of animals other than the guinea pig.

The following findings of this study are noteworthy: first, the pattern of somatostatin-like immunoreactivity by molecular forms showed that guinea pig visceral tissues do not conform to the broad patterns found in most mammals (4,17,18,20-22); second, these tissues exhibited significant quantities of H_2-S14, a peptide previously described only in guinea pig brain structures; and third, these tissues all contained significant levels of dihydrosomatostatin-28, which has not been reported in any species or tissue previously.

It is well established in the somatostatin literature that there are two sources of SLI in peripheral tissues: endocrine cells, typified by pancreatic D-cells, are found in pancreatic islets and in gastrointestinal mucosa; and somatostatinergic neuronal elements in pancreas as well as GI musculature and submucosa. Neuron terminals are diffusely distributed throughout GI tissue on a roughly equal basis, whereas gastrointestinal D-cells are concentrated in the

antral mucosa, and in the mucosa of anterior regions of the small intestine, becoming scarce in the colon and rectum (46). Pancreas, of course, has a high density of both sources of SLI (3,21,102).

With the exception of pancreatic and antral D-cells, which secrete S14 almost exclusively, somatostatin- secreting endocrine cells in peripheral tissues tend to synthesize and secrete S28, primarily; neural SLI, on the other hand, is mainly of the S14 form. (17,46,94,98, 100,101). Although it is not certain why these differences in molecular forms of SLI exist with respect to source tissue, there is evidence pointing to the mechanism that leads to the diversity: endocrine cells apparently possess processing enzymes that yield S14 from proS by way of S28 as an obligate intermediate step; neural tissues, however, appear to utilize mechanisms that allow direct conversion of proS to S14, bypassing the S28 intermediate substrate altogether (99).

Evidence from the rat (17) clearly shows S14 to be present in all gastrointestinal tissues as well as pancreas. Somatostatin-28 is found in very small quantities in pancreas and stomach, but in much larger quantities in small intestine and colon. Still, in all rat GI tissues except jejunum, whole tissue S14 levels are higher than S28 levels. SLI from small intestinal mucosa had much higher S28 than S14 levels, whereas muscle tissue exhibited greater S14 than S28 levels throughout the gastrointestinal tract. This pattern of distribution of molecular forms of SLI is consistent with the known biosynthetic predilections of somatostatin-secreting cells in gut tissues. Small intestine, possessing an abundance of both endocrine

cells and somatostatinergic nerve terminals, exhibits significant quantities of both S14 and S28; but as endocrine cells become less common toward the distal ends of the colon, and since innervation by somatostatinergic terminals is essentially unchanged throughout the length of the GI tract, S14 should be expected to predominate in colonic tissue extracts.

It is highly probable that these distribution patterns for the molecular forms of somatostatin have a basis in functional considerations. In pancreas, somatostatin is a vital part of the insulin-glucagon balance; its inhibitory effect on both glucagon and insulin secretion is essential to the regulation of blood glucose homeostasis. Mammalian pancreatic tissues secrete S14 almost exclusively, and apparently they are at the same time many times more sensitive to S14 than to S28 (17,42,53). Similarly, S14 is the form of SLI primarily secreted by gastric parietal cells and by somatostatinergic neurons in the stomach muscle wall; it serves to inhibit gastrin and acid secretion, gastric motility, and gastric transit (44-47). Why these tissues secrete S14 rather than S28 is, however, a mystery. Granted, receptors on the cells targeted by these somatostatin secretions preferentially bind S14 over S28, so secretion of S14 is biologically economical; but this does not really answer the question. Did S14 become the hormone of choice because of evolution of an S14 receptor in this tissue, or did the receptor adapt to a preponderance of the 14-residue peptide? Or, did they become established simultaneously in response to other stimuli affecting their function? In the case of gastric somatostatin, this

latter possibility seems quite plausible, considering that exocrine secretions of this organ are affected by gastrin, gastrin releasing peptide, bombesin, possibly VIP and GIP, as well as cholinergic elements (44).

The same questions can be applied to S28 secretion by intestinal mucosal and epithelial D-cells. For some reason S28 seems to be more efficacious than S14 in regulating gut epithelial function (including inhibition of the absorption of monosaccharides, amino acids, calcium, and lipids, and the secretion of chloride ions by crypt cells [44,47,50]), and is secreted in higher proportion than S14 in these tissues. Again, it is not clear why S28 has been naturally selected to serve as the dominant type of SLI in regulation of epithelial function.

If these questions are not answerable for the two major forms of biologically active somatostatin, it is unlikely that the interactions of four molecular forms in guinea pig tissues will be easily explained. The presence of S14, S28, H_2-S14, and H_2-S28 suggests the possibility of a multiplicity of functions for different molecular forms of SLI that is of even greater complexity than that inferred in other mammals. The assumption that organisms develop biochemical mechanisms along given lines in order to gain an advantage--in terms of natural selection--leads to the hypothesis that a specific and unique function could exist for each of these four peptides, or they would not be found in such quantities. Maintenance of redundant pathways in an organism's biochemical lexicon does not necessarily give it a selective disadvantage, but it

is unquestionably biologically uneconomical.

The results obtained in the present study did not elucidate possible roles for any of the four molecular forms of SLI discussed above, but they do serve at least to establish the existence and quantities of somatostatin-14 and -28 and dihydrosomatostatin-14 and -28 in guinea pig peripheral tissues.

None of the tissues studied in the guinea pig deviated drastically from other species with respect to the predominant form of SLI: S14 was the major form in the pancreas, stomach, ileum, and colon, as is the case in the rat and other species (17); S28 was the major form found in duodenum, and S28 levels were generally high in all intestinal tissues, which again compares favorably with the rat.

However, a number of significant differences between rat and guinea pig can be seen. Perhaps the most striking departure was that in no guinea pig tissue did a single molecular form predominate to the exclusion of other forms. All five forms of SLI (grouping all forms of somatostatin precursors as one of these five forms) were found in all tissues studied, including the pancreas. In the rat, more than 99% of total SLI is in the form of S14 (17). Another striking difference noted was the high contribution of S28 to total SLI in intestinal tissues. In the rat, S28 accounts for approximately 27%, 28%, and 12% of total SLI in duodenum, ileum, and colon, respectively (17). In the same tissues of the guinea pig, S28 accounts for 41%, 32%, and 28% of total SLI. It should also be taken into consideration that the rat studies cited used gel filtration techniques, which have lower resolution than the chromatography used

in this study; thus immunoreactivity reported as S28 would not be distinguishable from that of H_2-S28. This being the case, S28 levels found in the rat should not be compared simply to levels of S28 in the guinea pig: guinea pig S28 and H_2-S28 should be combined to provide a more equitable basis for comparison. If the two forms are combined for this purpose, guinea pig S28-like immunoreactivity represents 45%, 46%, and 36% of total SLI in the duodenum, midgut, and colon. As a percentage of total SLI, then, the proportion of S28 in guinea pig gut is nearly twice as high as that found in the rat.

Without knowing for certain whether this S28/H_2-S28 immunoreactivity is secreted by neural or endocrine cells, or whether the cyclic and reduced forms are secreted in concert or separately, it is difficult to speculate as to the reasons for this relatively high percentage of total SLI. But if the generalizations described above hold true in the guinea pig and S28/H_2-S28 is secreted primarily by mucosal D-cells rather than neural elements, this could indicate a much more important role for somatostatin in regulation of gut mucosal and epithelial function in this species than in the rat. It is particularly interesting to apply this conjecture specifically to the colon, where S28/H_2-S28 represented three times the percentage levels found in the rat, and where dihydrosomatostatin-28 accounted for a full 15% of total immunoreactivity (Table II).

Of further interest, the percentage contribution to total somatostatin-like immunoreactivity of H_2-S28 appeared to increase distally in the gut, which is directly opposite to the pattern of decreasing S28 immunoreactivity for these tissues in both the guinea

pig (Table II, Fig. 14) and in the rat (17). This suggests an increased specific requirement for dihydrosomatostatin-28 in the regulation of gut function. Since the percentage contribution of S28 does not parallel the gradient exhibited by H_2-S28, the inference can be drawn that the function of H_2-S28 is possibly quite distinct from that of S28. Dihydrosomatostatin-14 and its cyclic counterpart, S14, also exhibit a somewhat opposite relationship in their contributions to total gut immunoreactivity. It differs from the S28/H_2-S28 situation, however, in that the cyclic peptide increases in percentage contribution distally (37%, 42%, and 57% in duodenum, midgut, and colon, respectively), whereas reduced somatostatin-14 displays a very low profile that tends to generally decrease distally (Fig. 14). These trends would appear to imply different roles for these two forms of the peptide as well. The markedly higher quantities of H_2-S14 in pancreas and stomach (12% and 10% of total SLI, respectively) are sufficient to suggest that the reduced tetradecapeptide plays a significant role in some aspect of regulation of these two organs, but that it is considerably less important in intestinal tissues.

It is important to note that there is not a simple correlation between levels of cyclic S14 and S28 and their reduced analogs. For example, in pancreas, S28 and H_2-S28 levels were almost equal; yet in duodenum, where H_2-S28 made its lowest contribution of all tissues studied, S28 was highest. Again, S14 was lowest in duodenum, but H_2-S14 was lowest in midgut (Fig. 14). This evidence argues against the hypothesis that H_2-S14 and H_2-S28 are merely metabolites or

nonfunctional byproducts of S14 and S28 biosynthesis. In either case, accumulation of sufficient reduced peptide to be measurable by RIA would indicate an accumulation of large quantities of an essentially useless compound, and it seems probable that the levels of the reduced peptides would exist in quantities reflecting a fixed percentage of the appropriate cyclic peptide in any tissue. Because the data do not show a fixed ratio for S28:H_2-S28 or S14:H_2-S14, there is some justification for postulating that the reduced peptides are synthesized purposefully in predetermined quantities to fulfill a specific biological function or functions.

If it is possible that reduced S14 and reduced S28 are deliberately synthesized factors with true biological activity, the nature and importance of the disulfide bridge between Cys-3 and Cys-14 needs to be examined in some detail.

The importance of specific three-dimensional conformation of peptides and proteins with respect to their function is unquestioned. Recognition of peptide hormones by target-cell receptors is thought to involve parameters of binding at least as strict as those governing enzyme-substrate interaction.

The relationship between peptide hormone conformation and biological activity requires examination of a number of considerations. At the simplest level, changes in primary structure such as increased or decreased chain length or substitution of hydrophilic residues for hydrophobic ones, for example, can have a dramatic effect on such properties as solubility in blood, which in turn will affect blood and interstitial fluid concentrations of the

hormone. More dramatic effects may also be brought about by amino acid substitutions that are sterically or functionally divergent from the native sequence. For example, insertion of a proline residue will have the steric effect of producing a sharp kink in in the peptide chain that will disrupt any extant alpha or beta conformations, or for shifting a critical side-chain out of the binding site of the peptide. Another example is the substitution of threonine for Ser-195 in the active site of chymotrypsin: both amino acids possess a side chain consisting of a hydroxyl group on an aliphatic chain, but threonine's structure differs sufficiently to alter the position of the functional group within the active site such that the electron transfer essential to enzyme function is barred, and enzyme activity is lost.

Many substitutions, of course, can occur without affecting the conformation of the peptide molecule's critical regions. Substitutions that are isosteric (e.g., leucine for isoleucine) or isofunctional (e.g., lysine for arginine or aspartic acid for glutamic acid) are generally well tolerated in any region of a peptide except for the most critical sequences. In some instances, substitutions that are neither isosteric nor isofunctional occur, but the changes in conformation induced thereby have little or no impact on the shape of the binding or active site of the hormone. Somatostatin-28 exhibits a number of such changes (Fig.1), such as Leu-8 in anglerfish S28 for Met-8 in mammalian S28. Such substitutions are considerably less common in the sequence of S14, leading to the postulate that the amino acids of the S14 sequence are

more critical to correct conformation of the binding site of the hormone, and less likely to be altered or substituted without sacrificing activity. A quantitative example of the risk of substitutions in the critical region of a small peptide is found in studies of synthetic analogs of the nonapeptide hormone oxytocin: by subtituting for Ile-3 with leucine, valine, and alloiso- leucine, uterine activity is reduced to 1%, 11%, and 6%, respectively, of native peptide activity.

In small peptide hormones such as S14, oxytocin, and vasopressin, then, it is not surprising to find that primary sequences differ very little from species to species and even from class to class. Because their primary sequences are so short, the probability that their critical sequences will be disrupted by even a single amino acid substitution anywhere in the molecule is much greater than in a larger hormone such as insulin.

The three-dimensional native conformation of a peptide is determined by the steric, electronic, and hydrophobic interactions of the side-chains of its component amino acids. This conformation is stabilized by a variety of attractive forces or effects between these side-chains, such as salt bridges, electrostatic interactions, hydrogen bonding, and hydrophobic effect. Current theory of protein and peptide conformation holds that the native three-dimensional structure is established as the nascent polypeptide chain extrudes from the ribosomal complex: the structure attained is therefore the result of optimization of enthalpic and entropic considerations such that the molecule resides in its lowest possible energy state short

of disruption of its molecular integrity.

In some peptides and nearly all proteins this three-dimensional character, or secondary structure, is further stabilized by the formation of disulfide bridges, covalent linkages between cysteine residues brought into proximity by the native conformation. Typically, disulfide bonds form in association with reverse or beta-turns in the peptide. Amino acid conservation of both beta-forming sequences and of cysteines at the end of such sequences is very high (37). Whether beta-turns are strictly necessary for disulfide bridge formation is debatable, but the apparent correlation suggests a functional relationship between β-forming sequences and cystines. Somatostatin is no exception to this correlation between secondary and tertiary structure: using Chou and Fassman's prediction method (37), the probability of -structure formation of the sequence at the C-terminus of S14 is quite favorable, and this sequence is quite highly conserved (the probability of β-structure formation by the sequence Thr-10/Phe-11/Thr-12/Ser-13/Cys-14 is 1.14, which is significantly higher than the 1.05 threshold value for predicted β--structure formation; also, the probability of alpha-structure formation is 0.86, well below the 1.05 mark).

It is obvious that disulfide bridges, being covalent linkages, lend superior stability to peptides when compared to the relatively weak intermolecular forces stabilizing the peptide's conformation. In peptides such as insulin, where two discrete peptide chains are linked by two disulfide bonds, the covalent linkage is indispensible to the molecule's integrity. But in smaller peptides such as

somatostatin, the function of the linkage is less clear. A survey of the known peptide hormones containing 50 residues or less quickly disabuses one of the notion that Cys-Cys linkages are common. Clearly, the biological activity of peptide hormones cannot be considered generally dependent upon covalent bond stabilization of their conformation; too many of them function quite happily without Cys-Cys bonds. Yet for those that do contain a disulfide bridge, biological activity may be quite tightly linked with preservation of tertiary structure.

Experiments performed with reduced somatostatin and other analogs have shown that, _in vitro_ and _in vivo_, H_2-S14 has been reported as being roughly equipotent with S14; the reduced form only produces about half of S14's maximal response, but it appears to maintain its effect twice as long(87). Non-bridged S14 analogs that lacked the ability to cyclize such as [Ala-3,14]-S14 and [SMeCys-3,14]-S14 exhibit drastically reduced activity, however (2% and 0.6%, respectively, _in vivo_ [86]). It has been suggested that these results prove that the high activity of reduced somatostatin is due to its rapid oxidation under bioassay conditions to yield the cyclic peptide, since removal of the cyclization option apparently destroys activity.

An alternative explanation for the failure of these non-cyclizable analogs to exhibit bioactivity rests on the principle discussed earlier: that tertiary structure does not determine conformation of a peptide, but only serves to stabilize already-existing native conformation. Before declaring the disulfide bridge

to be the heart of somatostatin's bioactivity, it would be appropriate to examine the nature of the analogs used in probing the bridge's function: in both cases, the Cys residues were not replaced in the most conservative manner, sterically or functionally. The [Ala-3,14]-S14 substitution replaces cysteines (hydrophilic) with alanine residues (hydrophobic), a step that could alter the peptide's solvation behavior in polar solvents, thereby affecting conformation. Further, substitution of Ala for Cys considerably reduces the probability that a beta-turn will be formed in the C-terminal region of the peptide (the difference between P_{α} and P_{β} drops from 0.28 to 0.07); this suggests that enough of a steric change could take place by virtue of the substitution to seriously alter the peptide's conformation, thereby inhibiting hormone-receptor interaction. Similarly, methylating the sulfhydryl groups in the second analog would markedly reduce the hydrophilicity of the cyteine residues; this, coupled with the increased likelihood fo steric hindrance brought on by the greater bulk of the methylated side-chains, could again alter three-dimensional conformation and in this fashion seriously affect biological activity of the analog.

It must be remembered that somatostatin is a highly conserved molecule in most vertebrates, and in lower species as well. Such conservation of primary sequence suggests that substitutions, however small, may have an effect on the molecule that is deleterious to species survival; otherwise, we would likely see more phylogenetic variation in the peptide. For this reason, analogs chosen to probe the role of any component of the somatostatin molecule should be

chosen with extreme care to avoid producing unlooked-for effects. One possible choice for probing the cyclization question would be [Ser-3,14]-S14, for instance; this analog would be sterically and functionally quite conservative, and would not be as likely to induce unwanted conformational changes as the two analogs cited above.

An elegant study of the conformation of somatostatin has been performed by D.F.Veber's group (113-115). These investigators found that somatostatin probably exists as an equilibrating mixture of at least four conformers, one of which is much more active than the others. They were able to use this hypothesis in designing a 6-residue superagonist to somatostatin (Pro/Phe-7/[D]-Trp-8/Lys-9/ Thr-10/Ala-11) that is 2-5 times more active than the native peptide. This analog's structure suggests that the critical sequence of somatostatin--residues 7 through 10--can be artificially held in the single best conformation, and that the other possible conformations are less important to biological activity. In the native peptide, it is argued that the Cys3/Cys-14 bridge is essential to activity, but only insofar as it stabilizes the existing conformers: [D-Cys-14]-S14 is fully bioactive, suggesting that the Cys residue itself is not a functional component of the binding site of the hormone. The hexapeptide superagonist confirms this view by virtue of its heightened activity without possessing either a Cys residue or a disulfide bridge.

The implications of this work with respect to H_2-S14 and H_2-S28 as bioactive agents are not completely clear. However, it would seem reasonable to suggest that dihydro-somatostatin also exists as a

mixture of conformers in equilibrium, but because the disulfide bridge is absent, the number of conformers is greater. If this was the case, we could expect dihydrosomatostatin to exhibit lower activity than S14, since it would exist as the most active conformer less often. This is in fact what bioassays of the activity of H_2-S14 have shown.

Brazeau and associates have suggested that somato-statin's disulfide bridge is essential to hold the peptide in a conformation that will interact favorably with the receptor, therefore biological activity is predicated upon the cystine bridge's integrity. Veber's work can be used to modify this view to state that the bridge serves mainly to increase the probability that the peptide will be in its most active conformation at a given point in time, and thereby increases the hormone's apparent biological activity. By this line of reasoning it is possible that H_2-S14 and H_2-S28 could be biologically active in their own right, without needing to cyclize, although their activity would be considerably lower than that of S14 and S28.

At this point, then, it might be prudent to refrain from making the assertion that disulfide bridges--in somatostatin, at least--are essential to biological activity. There can be little doubt, however, that the disulfide bridge plays some important roles in peptide hormone functions. For example, a disulfide bridge can greatly reduce a peptide's lability to exopeptidase degradation, thereby increasing its biological half-life in blood. This is certainly true for the cyclic versus reduced forms of S14 and S28

(87). But the issue of the biological activity of endogenous reduced somatostatin and the role of the disulfide bridge in this activity (or lack thereof) cannot be said to be resolved at this time.

The existence of significant levels of reduced somatostatin-14 and -28 in guinea pig brain structures (29) and in peripheral tissues also has some bearing on the debate over biological activity of these peptides. The levels of the reduced peptides in this species have been shown to be valid, and not the product of an artefact in the methods used. As such, one is led to consider two possible explanations for these findings: first, that the guinea pig possesses a unique biochemical pathway for the synthesis or degradation of somatostatin that produces an unintentional pool of the reduced peptides as either a byproduct of synthesis or a rate-limited intermediate product of degradation; or second, that these tissues synthesize reduced somatostatin deliberately to fulfill a specific endocrine, neuroendocrine, or paracrine function.

The possibility cannot be dismissed that reduced somatostatin-14 and -28 are nothing more than partially degraded molecular forms of the active peptides, on their way to disassembly to their component amino acids. The linear peptides are more easily digestible by exopeptidases than are the cyclic forms because the N-terminus of the peptide is not hindered by the disulfide bridge, so it is entirely logical to assume reduction would be one of the first steps of degradation. Reduction seems to be necessary to accomplish this, since no endopeptidase hydrolysis of any of the peptide bonds in the ring has been recorded in the literature. (Veber's hexapeptide

superagonist is completely resistant to proteolysis for this reason, and is excreted intact by the liver [113-115].)

The most compelling argument against the hypothesis that H_2-S14 and H_2-S28 represent partially degraded metabolites is their quantity. The quantities reported in the present study would seem to indicate that a rate-limiting reaction in the putative degradative pathway follows the reduction step, so the reduced peptides accumulate before it. This doesn't fit the thermodynamic considerations, however: reduction of the covalent disulfide bond is likely an endothermic reaction, whereas hydrolysis of peptide bonds would release energy--once reduction occurs, and exopeptidases have access to the terminal amino acids of the peptides, the degradation should proceed rapidly. By this line of reasoning, we should expect to find evidence of pooling of des-[Ala^1-Gly-2]-S14, not H_2-S14 or H_2-S28. Since this is not what the evidence shows, the degradation hypothesis must not be accepted unequivocally.

There is also a possibility that the guinea pig, through mutation, has somehow lost the ability to convert all of the preprosomatostatin synthesized by the ribosomes into fully functional S14 and S28: it is conceivable that a certain proportion of the prohormone is reduced by accident, and either secreted in this variant form or shunted aside for later degradation. This hypothesis is at odds with the view of biochemical systems of living organisms as models of elegance and economy, of course, but considering the many biochemical anomalies known to exist in the guinea pig (22), it is not an impossibility. However, such a mutation might be expected

to yield a fairly constant proportion of reduced peptide to cyclic peptide, since the anomaly would presumably be the same in all somatostatin-producing tissues. The fact that no such correlation has been found in brain or in peripheral tissues may indicate, then, that this hypothesis is unfounded.

There remains, then, the second possibility: that H_2-S14 and H_2-S28 are biologically active forms of somatostatin synthesized and secreted deliberately to fulfill a specific regulatory function.

The nature of this role is a topic for conjecture. It is not out of the question that a processing option for H_2-S14 and H_2-S28 exists, especially when the multiple processing pathways for prosomatostatin are considered (Fig. 3). There are at least four identified cleavage sites in the preproS molecule, and there is evidence for N-glycosylation at one specific site of proS (56). It is known that some tissues process intact $proS_{1-92}$ directly to S14, while others follow processing route through S28 (17,99). Catfish islet $S28_{6-28}$ presumably is processed by a different mechanism than either of the above. Also, evidence exists for cleavage options in some tissues that yield $proS_{33-92}$ and $proS_{33-76}$ along the pathway leading to S14 or S28 (56). Given the diversity of pathways by which different species and tissues can produce biologically active forms of somatostatin, the possibility of another processing mechanism existing for reduction of the disulfide bonds of S14 and S28 does not seem so unlikely.

It is probable that dihydrosomatostatin is either oxidized or degraded almost immediately upon release into the blood: more than

(65); this finding implies that reduced somatostatins are unlikely to act in an endocrine mode, at least systemically. It is possible, however, that some cells secrete H_2-S14 or H_2-S28 into the blood or interstitium in situations where an effect is desired in tissues in close proximity to the secretory cells, and somatostatin's effects at distal sites would be counter-productive with respect to the desired paracrine or short-range endocrine effect. In such cases, rapid degradation of the hormone in the blood would be highly desirable, and a more rapidly degradable form of the hormone would have obvious utility.

Another possibility is suggested by the evidence indicating H_2-S14 has a diminished--but considerably prolonged--maximal effect on target tissues (87). This may indicate that dihydrosomatostatin is in fact inactive, but is secreted and then oxidized to form the active S14 molecule in the blood or interstitium. The cyclization, then, could be serving to prolong somatostatin's effect on a specific tissue or cell.

A third possible role for the reduced peptides could be in neurological applications. Dihydro-S14 and -S28 may serve well as inhibitory neurotransmitters or neuromodulators because of a lower maximal inhibitory effect than S14 or S28; since inhibition of action potentials or synaptic transmission occurs by hyperpolarization of neuronal membranes, a less severe hyperpolarization could conceivably be produced by the dihydro forms, so neurons could be more quickly able to recover full functionality than if the cyclic

peptides had been involved. These neurons could then have a shorter refractory period after receiving inhibitory input, and would therefore be more rapidly responsive to multiple inputs, both stimulatory and inhibitory.

In any case, further evidence will be required to establish reduced forms of somatostatin as biologically active factors. It is essential to prove that reduced S14 and S28 have biological activity in their own right, using rigorously controlled bioassays utilizing either appropriate noncyclizable analogs or incubation conditions that discourage oxidation of cysteine residues. It would certainly be interesting if receptors could be identified that preferentially bind the reduced peptides, or if it could be proven that binding of reduced peptides to a common somatostatin receptor produced a biological response differing from that of the corresponding cyclic peptide. Another line of research could be to attempt to discern the mechanism whereby S14 and S28 are reduced--whether it be via glutathione, as discussed in the introduction, or by some other means. If these goals can be reached--proving that the synthesis and biological activity of H_2-S14 and H_2-S28 are deliberate and specific--these reduced forms of somatostatin could become eligible for consideration as hormones.

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