INVESTIGATIONS ON SECRETIN AND ANALOGUES

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ABBREVIATIONS

Abbreviations have generally been used according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (104). All amino acids used, except glycine and sarcosine, are of the L-configuration unless otherwise stated. The proposals of M. Bodanszky, concerning the use of an Arabic instead of a Greek numeral preceding the word peptide, to indicate the length of a peptide and the use of the word segment instead of fragment, have been followed (105).

Ala : alanine
Arg : arginine
Asn : asparagine
Asp : aspartic acid
au : absorbance unit
Boc : t-butyloxycarbonyl
tBu : t-butyl
BSA : bovine serum albumin
Bzl : benzyl
CD : circular dichroism
CHA : β-(cyclohexyl)-alanine
DCCI : N,N'-dicyclohexylcarbodiimide
DCHA : dicyclohexylamine
DMF : N,N-dimethylformamide
GIP : gastric inhibitory peptide; glucose-dependent insulinotropic peptide
Gln : glutamine
Glu : glutamic acid
Gly : glycine
HCl : hydrochloric acid
HF : hydrogen fluoride
His : histidine
HOBt : 1-hydroxybenzotriazole
HPLC : high-performance liquid chromatography

i.m.	:	intra muscular
LAO	:	L-amino acid oxidase
LAP	:	leucine aminopeptidase
Leu	:	leucine
Lys	:	lysine
Nle	:	norleucine
NMM	:	N-methylmorpholine
NMR	:	nuclear magnetic resonance
Np	:	p-nitrophenyl
Nps	:	o-nitrophenylsulfenyl
ORD	:	optical rotatory dispersion
Orn	:	ornithine
Phe	:	phenylalanine
REMA	:	repetitive excess mixed anhydride
RIA	:	radioimmunoassay
RP	:	reverse-phase
Sar	:	sarcosine
Ser	:	serine
TFA	:	trifluoroacetic acid
Thr	:	threonine
TLC	:	thin-layer chromatography
Tos	:	tosyl (p-toluenesulfonyl)
Trp	:	tryptophan
Tyr	:	tyrosine
Val	:	valine
VIP	:	vasoactive intestinal peptide
Z	:	benzyloxycarbonyl

INTRODUCTION

Secretin is a hormone that is produced by a specific endocrine cell type in the duodenum and jejenum of many animals and man. Its most conspicious action is the stimulation of the pancreas to secrete a basic, enzymes-containing pancreatic juice. Secretin is secreted into the bloodstream on acidification of the contents of the upper part of the gut.

Secretin was first isolated from hogs. Porcine secretin appeared to be a linear 27-peptide amide. It was synthesized in this laboratory* via the repetitive excess mixed anhydride (REMA) method, in order to study the scope of this method in the synthesis of peptides in solution.

It seemed attractive to investigate the use of porcine secretin as a therapeutic agent for ulcers of the gastrointestinal tract or as a diagnostic agent for, e.g., the function of the pancreas. A big problem, however, was the instability of the peptide in vitro. A number of investigators had reported a rapid loss of potency, especially in aqueous solution. The cause of this phenomenon was not known.

The object of the investigations described in this thesis was to ascertain the cause of the rapid inactivation of porcine secretin in vitro at the molecular level and the finding of a practical remedy.

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1. THE GASTROINTESTINAL HORMONE SECRETIN

1.1 DISCOVERY, STRUCTURE ELUCIDATION, AND SYNTHESIS

In 1902, on January 16th, Bayliss and Starling discovered that the pancreatic secretion in a dog could be stimulated by introducing dilute hydrochloric acid into the jejunum from which all nerve connections with the body had been cut (1). They also discovered that an extract of the mucosa of the jejunum, after injection into the bloodstream, stimulated the pancreatic secretion. From these and following experiments they concluded that the stimulation of the pancreas was caused by a chemical messenger, that was transported from the jejunum to the pancreas via the bloodstream. They proposed the name "secretin" for the compound concerned. Soon this name had been generally accepted. This was the first time that body regulation via compounds, transported by the blood, was suggested. Later it would appear that many functions in the body are regulated in this way. The compounds concerned were called hormones. So, secretin was the first hormone recognized as such. Secretin was found in many animal species and in man.

It is interesting to recall here that the first person who succeeded in obtaining the pancreatic juice, in 1664, was the Dutch scientist Reinier de Graaf, who later lived in Delft as a physician (2).

The hormone secretin was first isolated from hogs. In 1961 Jorpes and Mutt isolated 10 mg of pure secretin from the upper intestines of 10,000 hogs (3). It was proved to be a linear peptide amide, consisting of 27 amino acid residues. In 1966 Jorpes and Mutt proposed the following sequence for porcine secretin (Fig. 1.1) (3):

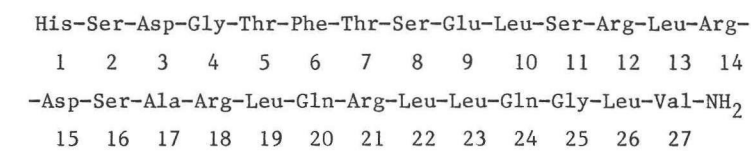

Fig. 1.1 The sequence of porcine secretin

The correctness of this sequence was proved in 1967 after the synthesis of a peptide with the proposed sequence by Bodanszky et al. (4). They obtained a product that was identical with natural porcine secretin. The peptide has since been synthesized several times. In Table 1.1 the successful syntheses are summarized.

Table 1.1 Syntheses of porcine secretin[a]

	Year	Strategy	Reference
Bodanszky et al.	1967	sequential synthesis[b,c]	4
Ondetti et al.	1968	fragment condensation[b]	5
Wünsch et al.	1972	fragment condensation[b]	6
Jäger et al.	1974	fragment condensation[b]	7
Van Zon and Beyerman	1976	sequential synthesis[b,d]	8
Yanaihara et al.	1977	fragment condensation[b]	9
Hemmasi and Bayer	1977	sequential synthesis[e,f]	10

a Several approaches didn't lead to a successful synthesis.
b Synthesis in solution.
c Coupling via active esters.
d Coupling via mixed anhydrides.
e Solid phase peptide synthesis.
f Coupling via the DCCI-method.

Mutt reported the isolation of bovine and chicken secretin (79). The isolation and characterization of chicken secretin has recently been described (107). It was found to be a linear 27-peptide amide, with great sequence similarities with porcine secretin (14 identical amino acid residues in corresponding positions). Mutt et al. isolated a peptide from extracts of porcine brain tissue that had chemical, pharmacological and chromatographic properties like porcine secretin (87). The sequence of this peptide has not been published as yet.

The conformation of porcine secretin in solution has been studied by several groups. After CD-, ORD-, and NMR-measurements on porcine secretin, partial sequences of porcine secretin, and analogues thereof, Bodanszky et al. (12-14) and Patel et al. (15) concluded that the peptide existed in a preferred conformation in aqueous solution. At first, Bodanszky et al. stated that the N-terminal part occurred as an α-helix, while the C-terminal part was necessary for the stabilization of this conformation. Later, also based upon

analysis of the sequence with the secondary structure conformational parameters of Chou and Fasman, they stated that the C-terminal part occurred as an α-helix, while a part of the remaining sequence was necessary for the stabilization of the conformation. Especially the side chains of the Glu^9- and Asp^{15}-residues should play an important role in the stabilization, probably via formation of ion pairs with the side chains of Arg-residues, present in porcine secretin in four positions. The N-terminal tetrapeptide did not influence the conformation. The conformation was changed by changing the temperature. Jaeger et al. (16) found that the conformation of porcine secretin in solution changed gradually on changing the pH from pH 6.8 to pH 2.0. According to CD-measurements lowering of the pH caused a decrease in the ordening of the peptide. These results support Bodanszky's hypothesis that ion pairs stabilize the conformation of secretin. Yanaihara et al. (17) found no significant dependence of the CD-spectra of porcine secretin on the pH in the range between pH 5.3 and 8.0. The three-dimensional polypeptide folding of secretin in aqueous solution has also been studied (18), using a combination of dark field electron micrographs, the parameters of Chou and Fasman, and a hierarchy of interaction types comprising the tertiary structure. The proposed structure contains four β-turns at tetrapeptides 1-4, 10-13, 13-16, and 16-19, β-sheet from residues 4-10, and α-helix from 19-27. This model is consistent with the data obtained by the other workers mentioned.

Porcine secretin has not been crystallized until now. Probably, the instability of the peptide in solution already mentioned, has played an important role. The conformation of secretin in crystalline form is therefore unknown.

1.2 THE BIOLOGICAL ROLE OF SECRETIN

Much has been done and is being done to elucidate the biological role of secretin. The hormone plays, to present knowledge, an important role only in the gastrointestinal tract of many animal species and man. Together with many other compounds it is important in the digestion of food, a very complicated process. It is difficult, if possible at all, to elucidate the exact role of secretin. The most important function would appear to be the stimulation of the pancreas to secrete a weakly basic, enzymes-containing juice. Table 1.2 summarizes a number of functions that are influenced by secretin. The most important stimulant for the release of secretin into the bloodstream is the acidification of the upper part of the gut. An extensive review of the investigations in this field is beyond the scope of this thesis. Here only some books or articles concerning the subject are referred to (19-24, 79).

Table 1.2 Biological activities that can be influenced by secretin (23)

1. Parotid
   Stimulation of fluid and electrolyte

2. Gastroesophageal sphincter
   Relaxation by competitive inhibition of gastrin

3. Stomach
   a. Inhibition of parietal cell secretion
   b. Relaxation of muscular activity
   c. Antitrophic

4. Duodenum
   a. Stimulation of mucous secretion by Brunner glands
   b. Relaxation of the sphincter of Oddi
   c. Relaxation of muscular activity

5. Pancreas
   a. Stimulation of fluid and electrolyte
   b. Synergistic facilitation of enzyme response to pancreozymin (cholecystokinin)
   c. Release of insulin
   d. Increase in blood flow

6. Liver and gallbladder
   a. Alteration of hepatic fluid and electrolyte secretion
   b. Stimulation of water and bicarbonate by liver ducts
   c. Relaxation of the gallbladder
   d. Increase in permeability of gallbladder mucosa

7. Small and large intestines
   a. Increase in mucosal transport of fluid and electrolyte
   b. Increase in secretion of fluid and electrolyte by intestinal glands
   c. Decrease in motor activity

1.3 NATURAL PEPTIDES, RELATED TO SECRETIN

A considerable number of peptides have been isolated from many animals and man. Elucidation of the primary structures showed that a number of peptides, isolated from the same species, possess great similarities in their sequences (21, 24, 25).

Fig. 1.2 shows the sequences of four peptides that have been isolated from hogs: the vasoactive intestinal peptide (VIP), secretin, pancreatic glucagon, and the gastric inhibitory peptide (GIP). Recently, a new member was added to this family (26). The sequence of this 27-peptide, named PHI by the discoverers (Peptide with N-terminal a His-residue (H) and C-terminal a Ile-residue (I)), has not been fully elucidated as yet.

Porcine secretin and chicken secretin have 14 identical amino acid residues in corresponding positions. The proposed sequence of chicken secretin is also shown in Fig. 1.2.

1.4 SYNTHETIC PEPTIDES, RELATED TO PORCINE SECRETIN*

Secretin, partial sequences of secretin, analogues of these, and elongated sequences of secretin have been synthesized by several groups. The synthetic peptides can be classified into groups, based upon the reasoning behind the synthesis:

1. Some peptides were synthesized with the intention of obtaining a biologically potent product that shows an protracted activity compared with secretin. This might be of practical importance for the application of secretin as a therapeutic or diagnostic agent.

2. In order to find the cause(s) of the inactivation of secretin already mentioned, several peptides were synthesized in order to investigate related physical, chemical, and pharmacological phenomena.

3. Some peptides were desired for studies on structure-activity relationships in gastrointestinal hormones.

4. Some peptides were synthesized in order to evaluate a radioimmunoassay (RIA) for secretin.

---

* The name "secretin" will be used henceforth, unless otherwise stated, when porcine secretin is meant. Often secretin will be abbreviated as S. Partial sequences will be named S(n-m), in which n and m indicate the place occupied in secretin by corresponding amino-acid residues, if His occupies position 1 and Val position 27 (see Fig. 1.1).

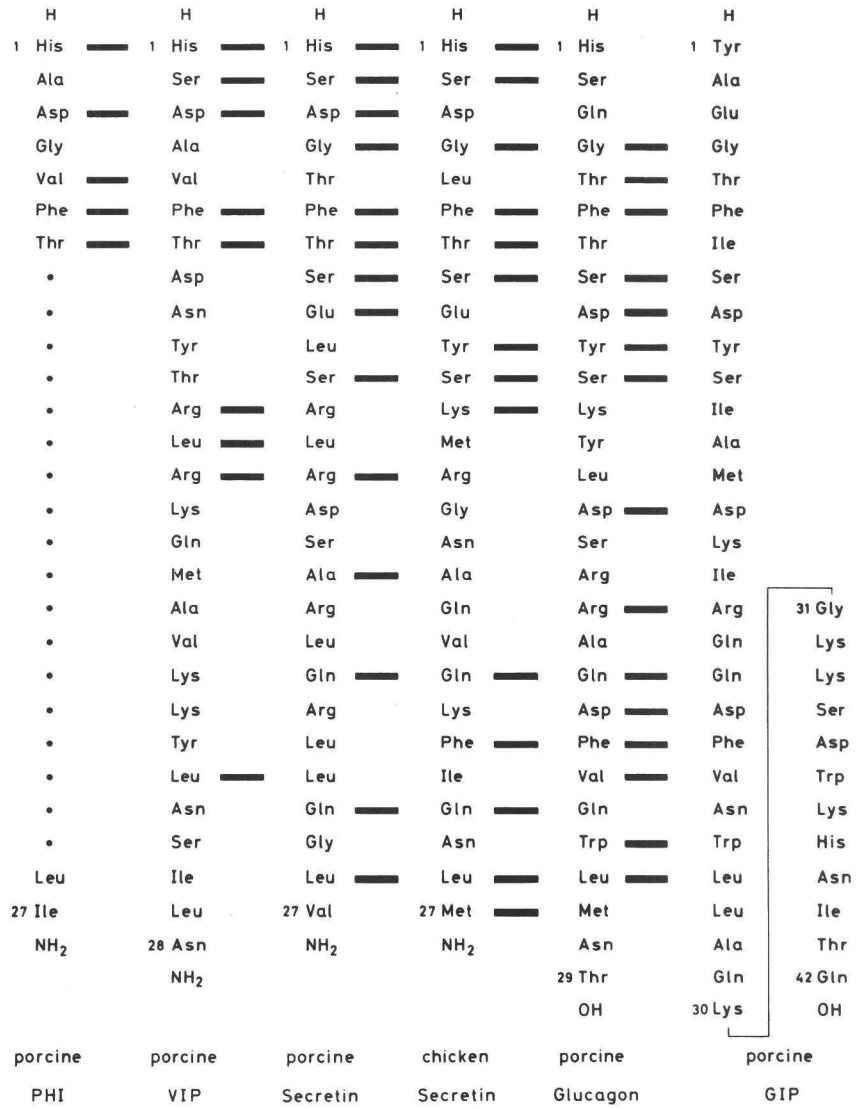

Fig. 1.2 The sequences of porcine VIP, porcine and chicken secretin, porcine glucagon, and porcine GIP. The horizontal bars indicate that the same amino acid residues are present in that position in neighboring peptides. The known amino acid residues in porcine PHI are also shown (see chapter 1.3).

Of course, this classification has no sharp boundaries. Some products might be synthesized for several of the reasons mentioned or might be used for various purposes.

A number of peptides have been synthesized by different groups. The pharmacological properties, always compared with pure secretin, were sometimes hard to compare. Methodological differences in the bioassays used could be responsible for this, as well as different qualities of the products.

In Table 1.3 a survey is given of synthetic peptides, that are related to secretin. This field has recently been reviewed by König et al. (27).

Table 1.3 Synthetic peptides, related to secretin

Peptide	Biological potency[a]	References
S(2-27)	low potency, 1[b]	12,28
various fragments	low potency	27,29-34,39,92
[$Orn^{12,14,18,21}$]-S	potent[b,e]	35
[Ala^{2}]-S	50[b]	27
[D-Ser^{2}]-S	9[b]	27
[β-Asp^{3}]-S	low potency, 1[b]	12,37
[Aspartoyl^{3}]-S	2[b]	16
[Asn^{3}]-S	1[b],7[d]	38,39
[Gln^{3}]-S	potent[b,f],0.1[b]	16,38
[Glu^{3}]-S	2[b],33-50[d]	38,39
[Ala^{4}]-S	2[b],100[d]	33,38,39
[D-Ala^{4}]-S	8[b],37[b],100[d]	17,38,39
[Pyr(3)Ala^{1}]-S	88[b]	17
[Ala^{4},Val^{5}]-S	potent[b,f],10[b]	16,33,84
[D-Ala^{4},Val^{5}]-S	11[b]	27
[Ala^{5}]-S	35[b]	27
[Val^{5}]-S	65[b],100[d]	27,39
[Tyr^{1},Ala^{2},Glu^{3}]-S	potent[b,f]	16
[$Phe^{1,2}$,Trp^{3},Lys^{4}]-S	potent[b,f]	16
[D-Trp^{4}]-S	5[b]	17
[Tyr^{10}]-S	potent[b]	40
[Gln^{3},Tyr^{10}]-S	potent[b]	40
[Glu^{3},Tyr^{10}]-S	potent[b]	40
[Ala^{4},Tyr^{10}]-S	potent[b]	40
[D-Phe^{6}]-S	19[b],low potency[b]	17,27
[CHA^{6}]-S	3[b]	27
[Ala^{11}]-S	80[b]	27
[desaminoHis^{1}]-S	25[b]	27
N_{α}-X-S X: various compounds	potent[g]	41
N_{α}-Gly-Gln-S	8[b]	36
N^{α}-Gly-Lys-S	3[b]	36
N^{α}-Gly-Gly-Gly-S	19[b]	36
[D-X]-S X: His^{1},Ser^{2},Phe^{6},$Arg^{12,14,18,21}$	potent[g] in various combinations	42
[Tyr^{1}]-S	4[b]	9
[Tyr^{6}]-S	1[b],potent[b,d]	9,44
N_{α}-Tyr-S	26[b],8[b]	9,27
N^{α}-desaminoTyr-S	4[b]	9
N^{α}-(desaminoTyr-β-Ala)-S		43
[Asp^{9}]-S(5-27)		14
[Gln^{9}]-S(5-27)	potent[c,d,f]	29
[Asn^{15}]-S(5-27)	potent[c,d,f]	29
[Gln^{9},Asn^{15}]-S(5-27)	potent[c,d,f]	29
[Lys^{15}]-S(5-27)	potent[c,d,f]	29
[Lys^{21}]-S(5-27)		106
[Asp^{9},Lys^{21}]-S(5-27)		106

a Only results of in vivo experiments have been given. The potency of the peptide to stimulate the exocrine pancreatic secretion was determined. This potency was compared with that of secretin. The biological potency of secretin was assumed 100. b Experiments with dogs. c Experiments with Guinea pigs. d Experiments with rats. e Experiments with cats. f Non parallel dose-response curves of peptide and secretin. g Protracted activity in comparison with secretin.

2 SYNTHESIS OF SECRETIN BY THE REPETITIVE EXCESS MIXED ANHYDRIDE (REMA) METHOD

2.1 INTRODUCTION

Secretin has been synthesized by Van Zon and Beyerman (8, 45) via the all-REMA method, as shown in Fig. 2.1a. Catalytic hydrogenolysis was used to remove all the protecting groups in one operation and purification was effected by ion-exchange chromatography. REMA-synthetic secretin proved to be highly potent in dog, cat, and rat bioassays for exocrine pancreatic secretion.

In the synthesis the following problems were met:

1. Several by-products were formed, according to the amino acid composition of intermediates, after acidic hydrolysis. Probably these by-products are "terminated sequences", as a result of coupling of the side-chain protected peptides with the undesired side of the mixed anhydride, the side of the auxiliary acid (Fig. 2.2). These products could be removed during the purification of secretin.

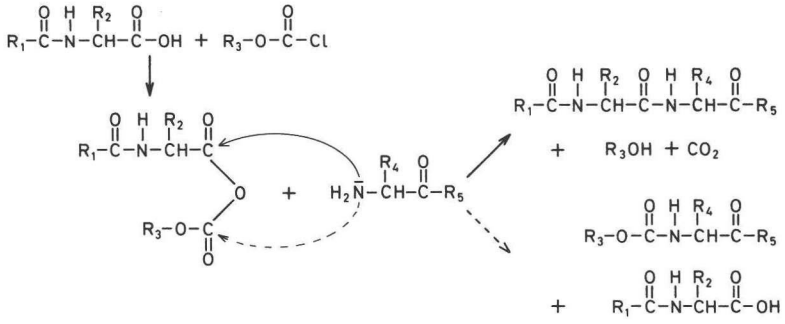

Fig. 2.2 The formation of the peptide bond (——) and the coupling with the auxiliary acid (----).

2. Secretin contains the sequence -Asp^3-Gly^4-. If secretin is synthesized as shown in Fig. 2.1, the sequence -Asp(OBzl)-Gly- occurs in the protected 25-, 26-, and 27-peptide. This sequence has a great tendency to form rapidly the

a

Deprotection	Amino acid derivative	Yield (%)[c]
H_2 Pd/C[d]	Z His Z	94
	Boc Ser Bzl	96
	Boc Asp Bzl	97
	Boc Gly	95
	Boc Thr Bzl	97
	Boc Phe	95
	Boc Thr Bzl	100
	Boc Ser Bzl	97
TFA	Boc Glu Bzl	100
	Boc Leu	99
	Boc Ser Bzl	96
	Boc Arg NO_2	97
	Boc Leu	93
	Boc Arg NO_2	99
	Boc Asp Bzl	85
	Boc Ser Bzl	89
	Z Ala	93
	Z Arg NO_2	94
	Z Leu	87
	Z Gln	98
	Z Arg NO_2	93
HBr/acetic acid	Z Leu	91
	Z Leu	89
	Z Gln	98
TFA	Boc Gly	97
	Z Leu	96
HBr/acetic acid	Z Val NH_2	90
		22[f]

b

Deprotection	Amino acid derivative	Yield (%)[c]
HF	Boc His Tos	89
	Boc Ser Bzl	96
	Boc Asp Bzl	96
	Boc Gly	93
	Boc Thr Bzl	94
	Boc Phe	91
	Boc Thr Bzl	100
	Boc Ser Bzl	100
	Boc Glu Bzl	98
	Boc Leu	98
	Boc Ser Bzl	98
	Boc Arg NO_2	98
TFA	Boc Leu	95
	Boc Arg NO_2	99
	Boc Asp Bzl	99
	Boc Ser Bzl	84
	Boc Ala	96
	Boc Arg NO_2	84
	Boc Leu	95
	Boc Gln	83
	Boc Arg NO_2	95
	Boc Leu	94
	Boc Leu	58[e]
	Boc Gln	92
	Boc Gly	98
	Z Leu	99
HBr/acetic acid	Z Val NH_2	95
		13[f]

Fig. 2.1 The syntheses of secretin by the REMA method

a Synthesis according to Van Zon and Beyerman (8, 45).
b Modified synthesis.
c Calculated on preceding protected peptide.
d Reaction time: 40 h; solvent: 80% aqueous acetic acid.
e During the coupling of Boc-Leu to the unprotected tetrapeptide probably <Glu-Gly-Leu-Val-NH_2 had been formed. This was removed during working up. If this step is ignored, the yield of crude protected secretin is 23%. In an independent synthesis of protected S(22-27)-NH_2 the couplings of Boc-Gln and Boc-Leu were performed with yields of 97%.
f Overall yield, calculated on the assumption that all products were pure, based on Z-Val.

sequence -aspartoyl-Gly-, both under acidic and basic conditions (45-50). Such a sequence can fall apart into a mixture of -β-Asp-Gly- and -α-Asp-Gly- (Fig. 2.3). Several investigators who used other coupling methods than the REMA method, were not able to obtain secretin or analogues thereof, because of difficulties in the last stage of the synthesis.

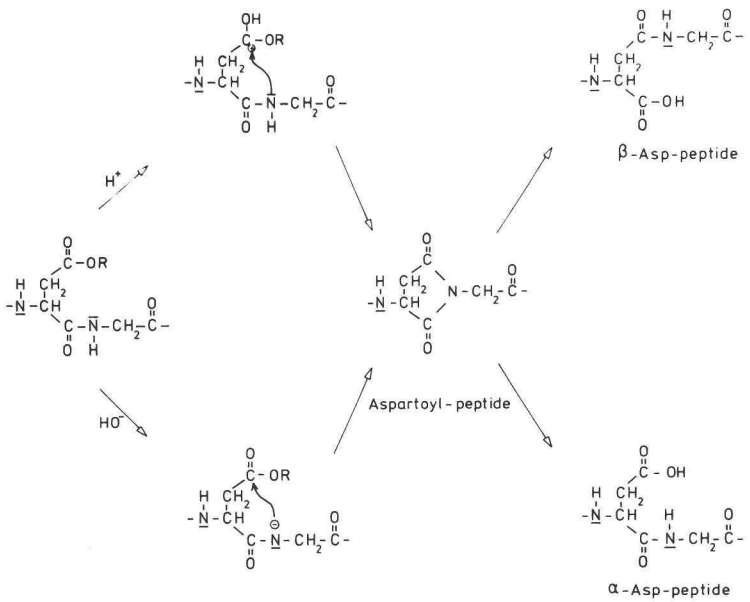

Fig. 2.3 The Asp(α→β)-Gly-rearrangement in peptides.

Van Zon found, in experiments with model peptides (45), that it was possible to prevent the formation of [aspartoyl]-peptides when the REMA method was used. It was not possible, however, to follow this reaction directly during the synthesis of secretin. Only after deprotection of the protected peptide could the possible formation of [aspartoyl]-peptide during the synthesis be determined, by amino acid analysis after enzymic hydrolysis by leucine amino-peptidase (LAP).

3. The removal of the protecting groups by catalytic hydrogenolysis sometimes gave rise to serious problems. In order to be sure that all protecting groups had been removed, the peptide material had to be exposed to the conditions used for a rather long time (24-48 h). Occasionally, the Phe^6-residue was found to be hydrogenated, and a cyclohexylalanine (CHA)-residue had been formed (up to 50% CHA). This phenomenon was not reproducible. The [CHA^6]-peptide could not be separated from the [Phe^6]-peptide.

Another side reaction during the final hydrogenolysis was the formation of a considerable amount of ornithine (Orn)-residues from Arg(NO_2)-residues. The by-products thus formed could be separated from the desired peptide in the final purification.

Van Zon monitored the synthesis of secretin by thin-layer chromatography (TLC) and amino acid analysis after acidic hydrolysis. Furthermore, in his synthesis the protecting groups were chosen in part so as to allow comparison of the physical constants of the intermediate products with those reported in the literature. For sequences longer than about 10 amino acid residues only the amino acid analysis gives valuable information. It was therefore desirable to use a better analytical technique for monitoring the synthesis. This became possible after the development of high-performance liquid chromatography (HPLC) systems.

Besides by hydrogenolysis, protected secretin can also be deprotected by acidolysis, e.g. in liquid hydrogen fluoride (HF). Hemmasi and Bayer used HF/anisole for the deprotection of protected secretin. After purification by ion-exchange chromatography, they obtained biologically fully potent secretin (10). Guiducci deprotected protected secretin in a similar way (51). These investigators did not mention side reactions. Wright studied the behaviour of protected secretin sequences in HF (30). He found that a fully active molecule was obtained after a short reaction time (30 min), while several by-products had been formed after longer periods. In all of the examples mentioned here secretin was synthesized on a solid support.

Several side reactions are known to occur as a result of the treatment of peptides with HF. The most conspicious reaction is the formation of [aspartoyl]-peptides from protected sequences with Asp-residues (Fig. 2.3). Especially the sequence -Asp(OBzl)-Gly- can undergo this reaction. Yang and Merrifield (47) found that a peptide with this sequence could give up to 99% of [aspartoyl]-peptide. The occurrence of this reaction greatly depends on the neighboring residues in the peptide sequence. Also the sequence -Asp(OBzl)-Ser(Bzl)-, present in protected secretin in Van Zon's approach, can give a considerable amount of a [aspartoyl]-peptide. We investigated whether the deprotection of protected secretin by HF is a suitable alternative for the hydrogenolysis.

The final purification of secretin has been performed in three ways: 1. Countercurrent distribution, in the system 1-butanol/fosfate buffer, pH 7.0 (1:1) (4, 5). 2. Ion-exchange chromatography with dilute NH_4HCO_3-buffers (6-10). 3. Gel filtration on Sephadex LH-20 with dilute hydrochloric acid (53). Some other methods of purification caused a considerable loss of biological potency (4, 53). HPLC looked a promising method for the final purification of secretin. Several other peptides had already been successfully purified by HPLC (54, 55). We studied the possibility of purifying secretin by HPLC.

Before the synthesis of a peptide can be considered as successful, it is necessary to have observed a satisfactory biological and immunological potency, in addition to chromatographic homogeneity in various systems and a correct amino acid composition after acidic and enzymic hydrolysis.

The determination of the biological potency of secretin was often troublesome. This was mainly caused by the rapid loss of the biological potency of secretin in dilute aqueous solutions. This phenomenon will be discussed extensively in chapter 3. In most cases the potency of the unknown samples to stimulate the exocrine pancreatic activity in animals is compared with the potency of a known sample, which had been compared with pure natural secretin. Several animal species, e.g. ferrets, Guinea pigs, dogs, cats, and rats, are used in the bioassays. Besides the stimulation of the pancreatic secretion other biological activities (Table 1.2) of secretin can also be used in bioassays.

It is possible to determine the immunological potency of secretin. At first the synthesis of radioactively labeled secretin, which is used in the radioimmunoassay (RIA), gave problems. This was caused by the fact that secretin contains no Tyr-residue, in which a ^{125}I-atom can easily be incorporated. In secretin the His-residue has to be iodinated. Several investigators have solved this problem and have developed a RIA (see for a recent review ref. 57). The immunological potency of the unknown sample is compared with that of pure secretin.

2.2 EXPERIMENTS AND RESULTS

Some minor tactical changes were introduced in the all-REMA synthesis of secretin, compared with the original synthesis of Van Zon and Beyerman (8, 45), as shown in Fig. 2.1. Also some new techniques for analysis and purification of products were developed. The points mentioned will be discussed consecutively in detail:

2.2.1 SYNTHESIS OF PROTECTED SECRETIN (1)

Protected secretin was synthesized as shown in Fig. 2.1b. The following changes were made in the choice of protecting groups, compared with the synthesis of Van Zon and Beyerman (8, 45):

1. Starting with Leu-Val-NH_2, the protected 27-peptide was assembled by using amino acid derivatives with the t-butyloxycarbonyl (Boc) group as N_α-protection exclusively, in order to simplify the procedure.
2. The N-terminal His was coupled as Boc-His(Tos), because of the better stability and accessibility as compared with the Z-His(Z) used first. Beyerman et al. had found that both derivatives could be coupled as mixed anhydrides without complications (56).

The synthesis of 1 was uneventful. The yields that were obtained were generally high (Fig. 2.1). The amino acid composition of this product is given in Table 2.1.

2.2.2 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) IN THE SYNTHESIS OF PROTECTED SECRETIN

The synthesis of protected secretin was monitored by reverse-phase HPLC (94). Methanol/water mixtures were used as the eluents and octadecylsilyl-silica was used as the stationary phase. Peptides containing the sequence -Asp(OBzl)-Gly- were chromatographed with eluents to which 1% of acetic acid had been added, because of the great tendency of such peptides to undergo an $Asp^3(\alpha\rightarrow\beta)$-rearrangement in methanol/water mixtures. This reaction did not occur when acetic acid had been added, which did not appreciably change the chromatographic behaviour. Fig. 2.4 shows the chromatograms of protected secretin and of rearranged protected secretin. In particular the possibility to monitor this $Asp^3(\alpha\rightarrow\beta)$-rearrangement was an improvement in the synthesis of secretin. This made it possible to synthesize [aspartoyl^3]-secretin, as will be described in chapter 5.

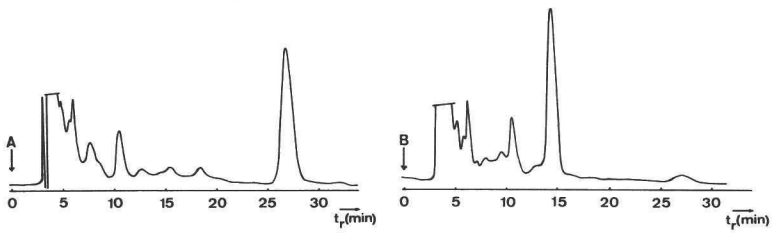

Fig. 2.4 Chromatograms of protected secretin and protected [aspartoyl^3]-secretin.

Protected secretin (1) was dissolved in DMF (1 mg/ml; A). NMM was added (10 µl/ml, pH ≈ 9). This solution was stored for 30 min at ambient temperature and analysed (B). Column: Polygosil C18, 10 µm (30 x 0.4 cm I.D.); eluent: methanol/water/acetic acid 93:7:1; UV-detection at 260 nm.

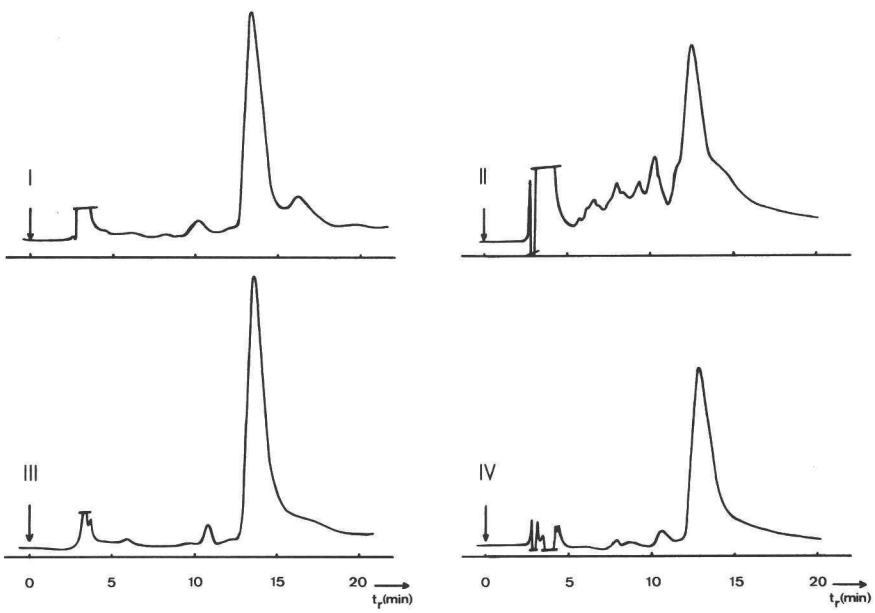

Fig. 2.5 Chromatograms of secretin.

I, II: crude unprotected secretin (2).

III, IV: purified secretin (3).

Column: Polygosil C18, 10 µm (30 x 0.4 cm I.D.); eluent: methanol/water/TFA 64:36:0.1 (I, III), methanol/water 82:18 with 0.005 M perfluorooctanoic acid (II, IV); UV-detection at 205 nm (I, III) or 215 nm (II, IV).

2.2.3 DEPROTECTION OF PROTECTED SECRETIN BY ACIDOLYSIS WITH LIQUID HYDROGEN FLUORIDE

Preliminary experiments showed that secretin, obtained after deprotection of crude protected secretin, synthesized as shown in Fig. 2.1a, had about the same biological potency after deprotection either by catalytic hydrogenolysis or by acidolysis with HF/anisole*. Amino acid analysis, after enzymic hydrolysis with LAP, showed that no [aspartoyl^3]-secretin had been formed after deprotection by either method.

Protected secretin (1) was treated with liquid HF to which 10% anisole had been added as an cation scavenger. The crude secretin (2) thus obtained possessed the same amino acid composition after both acidic and enzymic hydrolysis (Table 2.1). Consequently, no [aspartoyl^3]-secretin had been formed. This was confirmed by HPLC analysis, as described in 2.2.4. The formation of ornithine-residues had also been reduced, compared with the formation during the deprotection by catalytic hydrogenolysis.

2.2.4 ANALYSIS OF SECRETIN BY HPLC

It proved to be possible to analyse not only protected peptides, but also unprotected peptides by reverse-phase HPLC. Chromatography with methanol/water mixtures, to which perfluoroalkanoic acids had been added (lypophilic ion-interaction chromatography) gave excellent results on octadecylsilyl-silica as the stationary phase (95-97). Table 2.2 shows the k'-values of secretin in the systems that were routinely used for analysis.

The products of an Asp^3($\alpha \rightarrow \beta$)-rearrangment in secretin, [β-Asp^3]-secretin and [aspartoyl^3]-secretin could be separated in one of these systems (96). This will be discussed in chapter 5.

Chromatograms of 2 are shown in Fig. 2.5.

2.2.5 PURIFICATION OF SECRETIN

Crude secretin (2) was purified by ion-exchange chromatography on SP-Sephadex C-25, as described by Van Zon (45). The chromatogram obtained is shown in Fig. 2.6. Fraction 3 was analysed and appeared to be of good quality, according to TLC (> 95%, system B), HPLC (Fig. 2.5), and amino acid analysis after

* According to assays with the anaesthetized ferret (exocrine pancreatic response), performed by Professor T. Scratcherd, Sheffield, England.

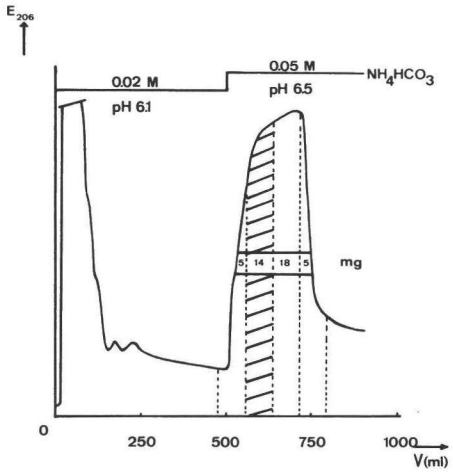

Fig. 2.6 The purification of secretin by ion-exchange chromatography. Column: SP-Sephadex C-25 (50 x 0.9 cm I.D.); eluent: NH_4HCO_3-buffers; UV-detection at 206 nm; flow rate: 15 ml/h. Crude secretin (100 mg), dissolved in 5 ml of water was purified at ambient temperature. The hatched area corresponds to the main fraction (3), the analyses of which have been given in the text.

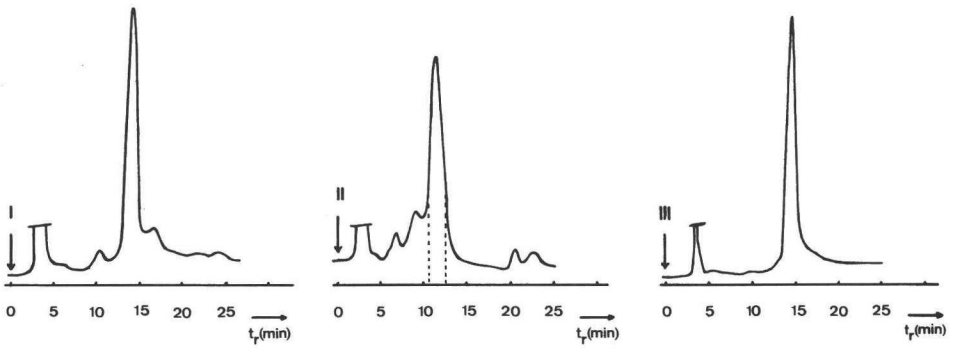

Fig. 2.7 The purification of secretin by HPLC.
I: crude secretin (4).
II: chromatogram of the purification of 4.
III: purified secretin (5).
Column: Polygosil C18, 10 µm (30 x 0.4 cm I.D.) (I, III), LiChrosorb RP-18, 10 µm (30 x 0.4 cm I.D.) (II); eluent: methanol/water/TFA 64:36:0.1 (I, III), methanol/water/TFA 67:33:0.5 (II); UV-detection at 205 nm (I, III) or 230 nm (II).

Table 2.1 Amino acid analyses of secretin

	1[a]	2[a]		3[a]		4[a]	5[a]	6[a]	7[a]	Theory
	b	b	c	b	c	b	b	b	b	
Asp	1.69	1.67	1.66	2.03	1.94	1.83	1.98	1.79	1.93	2
Thr	1.15	1.49		1.88		1.48	1.77	1.54	1.76	2
Ser	1.66	3.14		3.49		2.90	3.32	2.99	3.32	4
Glu	2.92	3.00	1.03	3.00	1.03	2.92	3.02	2.99	3.04	3(1[d])
Gly	1.82	1.79	1.82	1.97	1.94	1.74	1.84	1.83	1.97	2
Ala	1.12	1.08	1.13	1.04	1.00	1.07	1.12	1.09	1.10	1
Val	1.00	0.94	1.08	0.99	1.00	1.03	1.01	1.05	1.00	1
Leu	6.05	6.11	5.81	6.05	6.05	6.00	6.20	5.87	5.98	6
Phe	0.81	0.81	0.78	0.98	0.99	0.82	0.88	0.86	0.98	1
His	0.79	0.94	1.03	0.96	0.93	1.72	0.82	0.73	0.89	1
Arg+Orn	3.91	3.87	3.93	3.99	4.05	3.97	3.95	4.00	4.00	4

a See text.

b Acidic hydrolysis (6 M HCl, 24 h, 110 °C). Gln was determined as Glu.

c Enzymic hydrolysis (LAP, 24 h, 37 °C). Thr, Ser, and Gln could not be determined.

d Glu = 1; Gln = 2.

Table 2.2 Chromatographic data of secretin[a]

Ion-interaction reagent	Eluent methanol/water	k' [b]
0.1% TFA	64:36	3.6
0.005 M perfluorooctanoic acid	82:18	3.2

a Column: Polygosil C18, 10 µm (30 x 0.4 cm I.D.); temperature: 30 °C.

b $k' = (V_{peptide} - V_{water})/V_{water}$.

acidic and enzymic hydrolysis (LAP) (Table 2.1). This product was highly potent in a bioassay with the rat (Table 2.3). The other fractions obtained during the purification were of somewhat less quality (TLC, HPLC, amino acid analysis). One of these fractions had a very similar immunological behaviour as natural secretin (Fig. 2.8).

Crude secretin (4), obtained after HF/anisole treatment of protected secretin, synthesized as shown in Fig. 2.1a, was purified by HPLC on mg-scale (Fig. 2.7). The purified secretin (5) was practically pure according to TLC (system A, B) and HPLC (Fig. 2.7). The amino acid composition of 4 and 5, after acidic hydrolysis, is given in Table 2.1. The biological potency of 5, determined with the anaesthetized ferret, is shown in Table 2.3. A secretin sample, obtained after ion-exchange chromatography of crude secretin, was purified analogously, which also yielded a product (7) with a high biological potency (Table 2.3). The amino acid composition of the product before (6) and after (7) the HPLC purification is given in Table 2.1.

Table 2.3 Relative biological potency of secretin

Secretin	Biological potency (%)[a,b]	Animal	n[c]	m[d]
3	98 ± 8	rat	6	1
5	112 ± 10	ferret	1	5
7	95 ± 8	ferret	1	5

a See text (chapter 2.2.6 and 2.3.8). The biological potency of pure secretin was assumed to be 100%.

b After correction for peptide content.

c Number of animals used in an assay.

d Number of assays per dose in one animal. Only one dose was administered.

2.2.6 DETERMINATION OF THE BIOLOGICAL POTENCY OF SECRETIN

Secretin was assayed independently by two groups:

1. Professor T. Scratcherd et al., Department of Physiology, University of Sheffield, Sheffield, England determined the biological potency with the anaesthetized ferret.

2. Professor M.I. Grossman et al., Veterans Administration, Center for Ulcer Research and Education (CURE), Los Angeles, CA, USA, determined the biological potency with the anaesthetized rat.

Both groups determined the potency of the secretin sample to stimulate exocrine pancreatic secretion. Potency of the unknown sample was always compared with that of a known sample. The two groups worked differently:
1. Scratcherd and Hutson generally used one animal; occasionally two ferrets were used. In this animal a quantity of the unknown sample and a known sample were injected alternately. After each injection the pancreatic secretion was collected for 10 minutes and weighed. Equivalence between unknown and standard was established when intravenous injection of both gave identical or nearly identical responses.
2. Grossman and Solomon used a larger number of animals (generally 6 rats). In each animal the pancreatic secretion was collected and measured (15 min periods) after injection of the unknown sample and a known sample.

2.2.7 DETERMINATION OF THE IMMUNOLOGICAL POTENCY OF SECRETIN

The immunological potency of secretin was determined by Professor W.Y. Chey and Dr. T.M. Chang, The Genesee Hospital, The Isaac Gordon Center for Digestive Diseases and Nutrition, Rochester, NY, USA. The potency of the secretin sample to displace radioactively labeled secretin, [^{125}I-His^{1}]-secretin, from secretin antibody, obtained from rabbits, was determined (57). The potency of the unknown sample was compared with that of pure natural secretin. Fig. 2.8 shows a displacement curve of a fraction obtained after ion-exchange chromatography of 2.

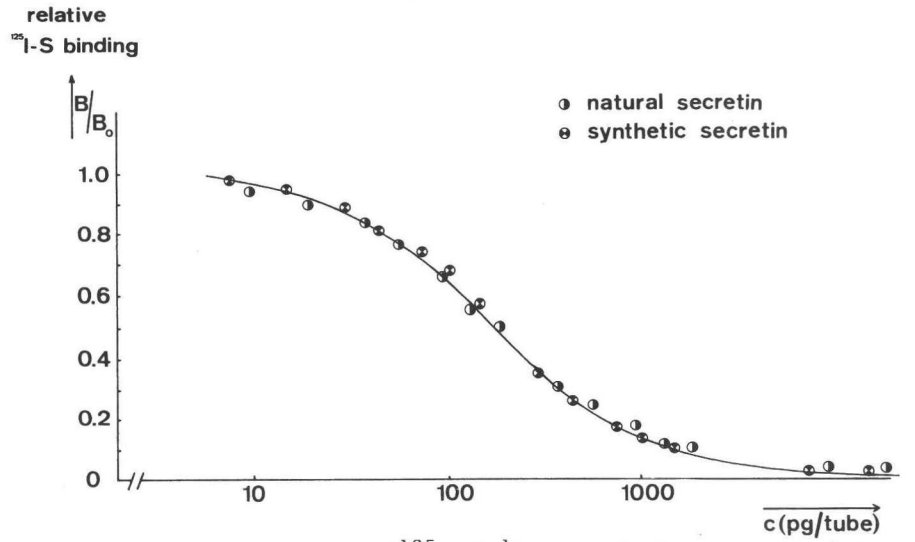

Fig. 2.8 The displacement of [^{125}I-His^{1}]-secretin from secretin antibody by natural and synthetic secretin

2.3 EXPERIMENTAL PART

Reagents, solvents, and amino acid derivatives were purchased and used without further purification, unless otherwise mentioned.

The acidolysis with HF was performed in an apparatus that was developed partly in this laboratory. The HF was distilled over CoF_3 before use.

For the analytical and preparative HPLC a Waters Model 6000 A pump with a Model U6K injector was used in combination with a Schoeffel Spectroflow Monitor SF 770 or a Pye Unicam LC 3 for variable wavelength detection.

2.3.1 SYNTHESIS OF PROTECTED SECRETIN BY THE REMA METHOD

Protected secretin (1) was synthesized as shown in Fig. 2.1b. The synthesis up to the 25-peptide was performed with 1.4 equivalents of the mixed anhydride. The synthesis of the 25-, 26-, and 27-peptide was performed with 2 eq. of the mixed anhydride.

A 1.4-fold excess of the mixed anhydride was obtained by adding 1.4 eq. of isobutylchloroformate to a solution of 1.5 eq. of N-protected amino acid derivative and 1.5 eq. of N-methylformaline (NMM) in dimethylformamide (DMF) at -15 °C. After 1-2 min, 1 eq. of the neutralized (by NMM) amino component, in DMF, was added to the activated amino acid derivative. The coupling was performed at -15 °C. The pH of the reaction mixture was maintained at pH 7.5-8.0. The progress of the reaction was followed by a spot test on silicagel with fluorescamine. After disappearance of 99.9% of the amino component, which was generally the case within a few minutes, the reaction mixture was stored for 1 h. Then, about 4 eq. of 2 M $KHCO_3$ were added and the solution was stirred for 15 min, while warming to 0 °C. The peptide was isolated by precipitation with water or saline, filtration, washing, and drying in vacuo.

The N-protecting groups were removed by acidolysis. The benzyloxycarbonyl (Z)-group was removed by treatment of the peptide with 2 M HBr in acetic acid for 90 min at ambient temperature. The Boc-group was removed by treatment with TFA or TFA/CH_2Cl_2 (1:1) during 30 min at ambient temperature. The N-protected peptides were isolated by evaporation of the solvent under reduced pressure, followed by trituration with ether, filtration, washing with ether, and drying in vacuo.

The yields of the coupling steps are shown in Fig. 2.1b. The overall yield of the synthesis, calculated on the assumption that all products were pure, was 13%, based on Z-Val.

2.3.2 DEPROTECTION OF PROTECTED SECRETIN BY ACIDOLYSIS WITH HF/ANISOLE

The experiments with HF were performed by Mr. P. Kranenburg.

Crude protected secretin (1, 213 mg) was treated for 1 h at 0 °C with anhydrous liquid HF (~ 10 ml) in the presence of anisole (~ 1 ml). After removal of the HF and anisole in vacuo, the crude reaction product was dissolved in 5 ml of 0.1 M acetic acid and the fluoride ions were exchanged by ion-exchange chromatography (BioRad AG 2-X8 (10 x 2 cm I.D.); acetate form) with 0.1 M acetic acid as the eluent. The fractions containing secretin were lyophilized. Yield: 190 mg of crude secretin. HPLC: see Fig. 2.5. Amino acid analysis: see Table 2.1.

2.3.3 PURIFICATION OF SECRETIN BY ION-EXCHANGE CHROMATOGRAPHY

Crude secretin (2, 100 mg) was purified on a column (50 x 0.9 cm I.D.), packed with SP-Sephadex C-25. The product was dissolved in 5 ml of water, put on the column, and eluted as follows: 1) 0.02 M NH_4HCO_3, pH 6.1 (500 ml), 2) 0.05 M NH_4HCO_3, pH 6.5 (450 ml), and 3) 0.10 M NH_4HCO_3, pH 6.9 (500 ml). The flow rate was 15 ml/min. Fractions were collected every 30 min. UV-detection was performed at 206 nm (Uvicord III). All fractions were lyophilized, followed by lyophilization from 0.1 M acetic acid. Yield: 42 mg. HPLC of the main fraction (3, 14 mg, peptide content: 68%): see Fig. 2.5. TLC: one major spot: R_f = 0.12 (system B). Amino acid analysis: see Table 2.1.

2.3.4 PURIFICATION OF SECRETIN BY HPLC

Crude secretin (4, 4.2 mg (peptide content 74%)) was dissolved in 0.22 ml of the eluent. Of this solution 4 x 0.4 ml was chromatographed on a column (30 x 0.4 cm I.D.), packed with LiChrosorb RP-18, 10 µm, in the eluent methanol/water/TFA 67:33:0.5 (see 2.3.5) at ambient temperature. The flow rate was 1 ml/min. The main fractions, according to UV detection at 230 nm (Fig. 2.7), were collected and lyophilized after the addition of 200 ml of water. After lyophilization from 0.1 M acetic acid, the yield was 1.7 mg of secretin 5 (peptide content: 57%; yield: 43%). HPLC: see Fig. 2.7. TLC: one major spot (system A, B). Amino acid analysis: see Table 2.1.

Analogously, 3.7 mg of secretin (6, peptide content: 88%) was purified. Yield: 1.6 mg of 7 (peptide content: 62%; yield: 30%).

2.3.5 ANALYSIS OF PEPTIDES BY HPLC

The eluents were mixed on a volume-to-volume basis and were degassed by careful filtration under reduced pressure, after which the ion-interaction reagent was added.

The protected peptides were dissolved in the eluent, or in DMF or acetic acid, after which an equivalent volume of the eluent was added. Underivatized peptides were dissolved in the eluent or in water. The concentration of the solutions was 0.1-10 mg/ml, of which 1-10 µl was injected.

The HPLC analysis were performed on a column (30 x 0.4 cm I.D.), packed with Polygosil C18, 10 µm. The column and the injector were maintained at 30 °C.

2.3.6 ANALYSIS OF PEPTIDES BY THIN-LAYER CHROMATOGRAPHY (TLC)

TLC was performed on silica gel plates (Merck silica gel 60 F-254) in the following eluents: A: Butanol/pyridine/acetic acid/water 30:20:6:24; B: Butanol/pyridine/acetic acid/water 60:20:6:24. The spots, after chromatography of about 10 µg of product, were made visible by the Reindel-Hoppe test after chlorination.

2.3.7 ANALYSIS OF PEPTIDES AFTER ACIDIC AND ENZYMIC HYDROLYSIS

Amino acid analyses were performed by Mr. A. van Estrik.

Acidic hydrolysis was performed in 6 M HCl for 24 h at 110 °C. The peptide content was determined by quantitative amino acid analysis, with norleucine (Nle) as an added internal standard.

The enzymic hydrolysis with leucine aminopeptidase (LAP, Sigma, St. Louis, MO, USA) was performed as follows: To 100 nmol of secretin, dissolved in 250 µl of 0.1 M 'Tris-buffer' (pH 7.8), 0.05 mg of LAP (13.2 u/mg) and 5 µl of toluene were added. The solution was shaken at 37 °C for 24 h. After lyophilization the mixture was analysed.

2.3.8 DETERMINATION OF BIOLOGICAL POTENCY

The biological potency of secretin was determined using rats or ferrets.

Male Sprague-Dawley rats, 200-350 g body weight, were fasted for 16 h. The rats were lightly etherized and injected i.m. with 0.25 ml/100 g body weight of a 25% solution of urethane in water.

A polyethylene (PE 50) catheter was inserted into the jugular vein and attached to a syringe. The pylorus was ligated. The bile-pancreatic duct was ligated proximally near the liver. A PE 50 catheter was inserted distally into the duct (outside the duodenum), and connected to an 0.1 or 0.2 ml glass pipet.

For bioassays the exocrine pancreatic secretion was determined at 15 min intervals. After two basal collections, doses were administered at 30 to 45 min intervals. Usually the following order of administration was performed: 1 ng/kg Squibb secretin, low unknown, 10 ng/kg Squibb secretin, middle unknown, 100 ng/kg Squibb secretin, high unknown.

The standard solution (synthetic secretin containing Cys.HCl (0.5 mg/mg peptide), Squibb, New Brunswick, NJ, USA) was prepared as follows: 1.8 mg of secretin was dissolved in 18 ml of water, containing Cys.HCl (1 mg/ml) and bovine serum albumin (10 mg/ml). Portions of 0.5 ml were lyophilized. For assay, dilution of 50, 5, and 0.5 ng/ml (in 0.154 M NaCl, containing BSA (10 mg/ml)) were made for doses of 1, 10, and 100 ng/kg. The "Squibb secretin" had been compared with pure natural secretin (GIH, Karolinska Institute, Stockholm, Sweden).

The unknown secretin sample was dissolved in 0.154 M NaCl (containing 10 mg/ml BSA). For assay dilutions were usually made of 50 ng/ml, 5 ng/ml, and 0.5 ng/ml.

The doses were injected and the catheter was then flushed with 0.2-0.3 ml of saline (with BSA).

The bioassays with the ferret were performed as described for the cat (58). The unknown secretin was dissolved in 0.9% NaCl, containing BSA (10 mg/ml). It was compared with the potency of crude natural secretin, the so-called "Boots secretin", prepared by Professor T. Scratcherd, according to the method of Crick et al. (69). This "Boots secretin" had been compared with pure natural secretin (GIH, Stockholm, Sweden).

2.3.9 DETERMINATION OF IMMUNOLOGICAL POTENCY

The immunological potency of secretin was determined as described in ref. 57.

2.4 CONCLUSIONS AND DISCUSSION

The synthesis of protected secretin via the all-REMA method again proceeded satisfactorily. Certain tactical changes would seem to be improvements. The use of the Boc group as N_{α}-protection and of the Tosyl group as N_{im}-protection simplified the synthesis. Starting with the tripeptide Boc-Gly-Leu-Val-NH_2, the synthesis of the protected 27-peptide, as to its performance, consists of 24 identical reaction stages. This simplifies mechanization, if used.

During the synthesis reverse-phase HPLC was used for the analysis of intermediates. This proved to be an excellent method for the analysis of protected peptides. The possibility to monitor the $Asp^3(\alpha\rightarrow\beta)$-rearrangement in protected secretin sequences, especially, is an important improvement. This rearrangement is probably the cause of difficulties reported during syntheses of secretin via other coupling methods. It proved to be possible to synthesize protected secretin by the REMA method, without the occurrence of the $Asp^3(\alpha\rightarrow\beta)$-rearrangement. The possibility to use reverse-phase HPLC in the purification of protected peptides will be shown in chapter 5.

The deprotection of protected secretin by liquid HF/anisole is a suitable alternative for the hydrogenolysis used previously. Thus the use of the N_{im}-Tos-protection becomes possible without the necessity of additional reaction stages. The HF-acidolysis involves no or hardly any formation of [aspartoyl]-peptide. This change in methodology enlarges the possibilities of synthesizing analogues of secretin, e.g. for investigating the structure-activity relationships.

Reverse-phase HPLC appeared to be very useful for the analysis of underivatized products in the synthesis of secretin. The $Asp^3(\alpha\rightarrow\beta)$-rearrangement could be followed in a simple system in secretin. This rearrangement was generally thought to be responsible for the problems that were met during the synthesis of secretin or during biological experiments with the hormone.

The purification of secretin by reverse-phase HPLC, with methanol/water/TFA as the eluent gave products with high biological potency. Amino acid analysis, however, showed that the product obtained was not pure.

The purification looks useful as an extra, final purification step, after purification by ion-exchange chromatography. This was performed in the synthesis of some analogues of secretin, as will be described in the chapters 5-7.

3. ON THE INSTABILITY OF SECRETIN

3.1. INTRODUCTION

Secretin has repeatedly been said to be unstable. On storage, especially in solution, its biological potency decreased.

During the purification of the first synthetic secretin, in 1966, Bodanszky et al. were seriously hindered by the instability of the peptide (4). During the purification by countercurrent distribution in the system 1-butanol/pyridine/acetic acid/water a considerable decrease of the biological potency was observed. Purification in the system 1-butanol/0.1 M fosfate buffer, pH 7.0 yielded a product with high biological potency. After analysis of a secretin sample with only 25% of the desired potency, obtained in the first system mentioned above, they concluded that an $Asp^3(\alpha\rightarrow\beta)$-rearrangement, but no $Asp^{15}(\alpha\rightarrow\beta)$-rearrangement had taken place. They remarked that these results might help to explain the instability of secretin.

Grossman reported, in 1969, that secretin rapidly lost potency on storage at -20 °C in rubber capped vials and was stable for at least 1 year when stored in all glass vials with cysteine hydrochloride added (59).

Spingola & Grossman stated, in 1973, that secretin was unstable at neutral pH at room temperature. Also in acidic solutions, secretin solutions should not be stored longer than several hours at 5 °C (60).

Wünsch et al. synthesized secretin in 1972. After purification by ion-exchange chromatography, with practically neutral dilute NH_4HCO_3 buffers as the eluent, followed by repeated lyophilization from dilute aqueous acetic acid, a pure product with high biological potency was obtained (6). In some papers, published in 1974 and 1979 (61, 62), Wünsch stated that, especially in weak basic solutions, an $Asp^3(\alpha\rightarrow\beta)$-rearrangement easily took place in secretin, which should be accompanied by a change in conformation and a loss of biological potency. Since then Wünsch' group has been continuously occupied with research on the cause(s) of the decrease of biological potency of secretin (16, 64, 65). In a number of papers, in 1977-1979 (16, 64), they reported that [aspartoyl^3]-secretin could be isolated from secretin after

storage of secretin in an aqueous solution (pH 3-4) for several weeks. From [aspartoyl^3]-secretin a mixture of secretin and [β-Asp^3]-secretin was rapidly formed in practically neutral solution. They followed the appearance and disappearance of signals ascribed to [aspartoyl^3]-secretin by a chiroptical method, circular dichroism. The biological potency of [aspartoyl^3]-secretin and [β-Asp^3]-secretin, determined in the dog, was low. They reported that pure secretin was fairly stable in neutral solution, but unstable in the presence of acid.

Geiger's group reported, in 1974, that secretin rapidly lost its biological potency in aqueous solution (7). The addition of cysteine hydrochloride did not much improve the stability. Pure secretin, in the acetate form, was at least for a short time stable as a lyophilized product. In later investigations, reported in 1977 (53), they obtained from crude secretin, obtained by deprotection of protected secretin, after repeated purifications by gel chromatography (Sephadex LH-20; 0.1% acetic acid) very pure secretin, according to TLC and amino acid analysis. This product, however, possessed only 20% of the desired biological potency. Similar results were also obtained after purification on silica gel with a mixture of chloroform, methanol, acetic acid, and water as the eluent. They found that the potency of secretin, dissolved in dilute acetic acid, decreased on prolonged storage. Purification of crude secretin by gel chromatography (Sephadex LH-20), with water or dilute hydrochloric acid (0.001-0.005 M) as the eluent, delivered a pure peptide with high biological potency. They concluded that secretin in the acetate form rapidly lost potency, while secretin in the hydrochloride form was considerably more stable. In another paper, in 1977 (38), they reported that secretin lost about 75% of its potency in slightly acidic solution and then lost no further potency. They did not succeed in determining differences by TLC and enzymic hydrolysis between fully potent secretin and secretin that had lost 75% of its potency. They expressed their doubts about the Asp^3(α→β)-rearrangement as the cause of the inactivation of secretin, also because of experiences with analogues of secretin.

Boden et al. investigated the loss of biological and immunological potency of secretin (66). They found that both natural and synthetic secretin, dissolved in very dilute, practically neutral solutions (pH 7.5, 25 ng/ml), lost its potency very slowly (maximum 50% decline after storage at -15 °C for 3 months).

Boden and Wilson reported (75) that purified ^{125}I-labeled secretin was stable for at least 4 days when kept in 0.1 M hydrochloric acid at 4 °C, according to chromatoelectrophoresis, while it retained its immunoreactivity

for 3 months if stored in acidified ethanol at -20 °C.

In commercially available natural secretin (GIH, Stockholm) the warning was enclosed that secretin, dissolved in sterile saline, loses its biological potency within hours (67).

During the determination of the biological potency of secretin, synthesized by Van Zon & Beyerman, in 1976 (8), it was found that 80% of its potency was lost within 3 hours on storage in neutral aqueous solution at 4 °C. The standard with which synthetic secretin was compared, was stable in solution for a long period (68). This standard was crude natural secretin, the so called "Boots secretin", prepared by Professor T. Scratcherd according to the prescription of Crick et al. (69).

Hemmasi & Bayer reported that the potency of secretin was lost on repeated lyophilization (10).

A number of additives have been proposed in order to increase the stability of secretin, e.g. cysteine hydrochloride, alanine, β-alanine. Several patents appeared, in which increased stability in secretin formulations was claimed. Some commercially available secretin preparations contain(ed) large amounts of cysteine hydrochloride (synthetic "Squibb secretin", natural "GIH secretin").

We studied the stability of secretin by different methods. Some potential causes of the decrease of biological potency of secretin are:
1. Microbiological degradation. This hardly explains any rapid decrease of biological potency. 2. Physical adsorption on materials during storage or while performing experiments. 3. Chemical reactions.

We approached the phenomenon of the decreasing biological potency as follows: 1. Characterization of the circumstances in which the potency decreases. 2. Studies on possible adsorption of secretin on glass. 3. Detection of intramolecular reactions in partial sequences of secretin and in secretin itself. 4. Synthesis and bioassay of analogues of secretin in which Gly^4 has been replaced by other amino acid residues.

3.2. LOSS OF BIOLOGICAL POTENCY OF SECRETIN

In bioassay experiments using conscious dogs, we found that solutions of secretin (pH 7.0; 250 ng/ml of 0.9% saline) did not lose potency significantly on storage at ambient temperature for 24 h. Secretin, stored at 25 °C for 72 h (pH 7.9, 0.5 mg/ml of 0.5% NH_4HCO_3), and then lyophilized, was not less potent than the starting material which was lyophilized immediately from dilute acetic acid. In independent experiments with anaesthetized ferrets, we found that solutions of secretin (pH 7.4, 1 µg/ml of 0.9% saline) rapidly lost most of their potency on storage at 22 °C. It proved impossible to arrive at reproducible figures concerning the decrease of potency in this system.

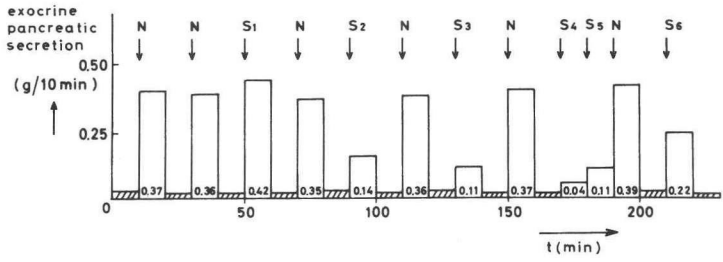

Fig. 3.1 Pancreatic secretion in an anaesthetized ferret after injection of aqueous solutions containing secretin (N: injection of natural secretin; S: injection of REMA-synthetic secretin).

The exocrine pancreatic secretory response during 10 min after injection and the basic response (hatched columns) were determined. The figures in the columns show the net response after injection (g/10 min).

REMA-synthetic secretin dissolved in water (c = 1.32 µg/ml) in a calibrating flask prepared of borosilicate glass (Pyrex, 10 ml), was injected, immediately after dissolving, into an anaesthetized ferret (0.4 ml; S1). After 40 min another 0.4 ml was injected (S2). After 70 min the solution was transferred to another flask prepared of the same material. This solution was injected after 50 min (0.4 ml; S4). The emptied flask was filled with a solution of BSA in 0.9% NaCl (10 mg/ml), and after shaking for 5 min this solution was injected (0.4 ml; S3). This was repeated after 50 min (0.4 ml; S5) and 80 min (0.8 ml; S6), respectively. Natural "Boots secretin" was used as a standard. This standard, in aqueous solution, was injected several times (N; 0.3 mg).

3.3. ADSORPTION OF SECRETIN ON GLASS OR SYNTHETIC MATERIALS

It is known that certain peptides in highly diluted aqueous solutions occasionally adsorb on the surface of their container (e.g. glass, synthetic materials) (70-74). Boden and Wilson reported that ^{125}I-labeled secretin adsorbed on glass. This adsorption could be prevented by the addition of bovine serum albumine (BSA) or serum to the peptide solution or by dissolving secretin in 0.1 M hydrochloric acid (75). Recently, adsorption has been observed with dilute secretin solutions (76-78). In order to prevent the adsorption, often some material is added to the solution, or the container surface is coated. Ogino et al. (78) found that the adsorption of secretin on glass could be prevented by the addition of BSA to the secretin solution. We studied the adsorption of secretin on glass (Fig. 3.1), and confirmed that secretin has a strong tendency to adsorb on this material. This adsorption was prevented by the addition of an excess of BSA to the solution. We found, indeed, in bioassay experiments with the ferret that no significant loss of potency occurred if an excess of BSA had been added to the solution and if the glassware which was used had been silylated. Ogino et al. (78) found, however, that silylation of the glassware increased the adsorption of secretin on the glass. In experiments with the rat we found that the potency of a secretin solution did not decrease on storage when only BSA had been added. It is likely that most of the observed rapid loss of biological potency is therefore caused by a reversible physical adsorption.

3.4. CHEMICAL REACTIONS IN MODEL PEPTIDES AND SECRETIN

We also studied the occurrence of some chemical reactions in secretin, potential causes for the decreasing potency. A contemplation of the sequence of secretin (Fig. 1.1) led us to several conceivable reactions (Table 3.1).

In literature the Asp^3($\alpha\rightarrow\beta$)-rearrangement (Fig. 2.3) has especially been mentioned as a possible intramolecular reaction in the peptide (4, 16, 38, 64, 65). We decided to study the Asp($\alpha\rightarrow\beta$)-rearrangement in secretin closely. In secretin two Asp residues are present, viz. Asp^3 and Asp^{15} (Fig. 1.1).

Table 3.1 Some conceivable reactions in secretin

Reaction type	Reaction site
Asp(α→β)-rearrangement	$Asp^{3,15}$
Glu(α→γ)-rearrangement	Glu^{9}
Deamidation	$Gln^{20,24}$, Val^{27}-NH_2
N→O shift	$Ser^{2,8,11,16}$, $Thr^{5,7}$
Lactone formation	Ser^{2}-Asp^{3}, Ser^{8}-Glu^{9}, Asp^{15}-Ser^{16}
Peptide chain cleavage	
Racemization	

First, we studied the behaviour of two segments of secretin, S(1-6)-NH_2 and S(13-18). In these peptides the immediate environment of the Asp residues is the same as in secretin. We synthesized a number of peptides: S(1-6)-NH_2, [β-Asp^3]-S(1-6)-NH_2, [aspartoyl^3]-S(1-6)-NH_2, S(4-6)-NH_2, S(13-18), [β-Asp^{15}]-S(13-18), S(13-14), and S(15-18). This will be described in chapter 4. Most of these peptides could easily be separated by reverse-phase HPLC with methanol/water/TFA mixtures as the eluent.

S(1-6)-NH_2 and S(13-18) were stored in 0.1 M ammonium acetate at different pH (50 °C, 65 h). The rather high temperature, 50 °C, was chosen to speed up the potential chemical changes and to minimize the microbiological degradation of the peptides. The solutions were then analysed by reverse-phase HPLC (UV detection at 205 nm), directly and after lyophilization followed by storage in 1% NH_4HCO_3 (ambient temperature, 24 h) (Table 3.2). Solutions, obtained in the same way were lyophilized, hydrolysed by LAP, and the amino acid composition was determined (Table 3.3). After storage of S(1-6)-NH_2 at pH 2.5 two main peaks were visible with reverse-phase HPLC: a peak with the retention of S(1-6)-NH_2 and [aspartoyl^3]-S(1-6)-NH_2, and a peak caused by S(4-6)-NH_2 (according to the amino acid composition of the collected product after acidic hydrolysis and comparison with synthetic S(4-6)-NH_2). After treatment of the lyophilized mixture with 1% NH_4HCO_3 a new peak appeared, with the retention of [β-Asp^3]-S(1-6)-NH_2. After storage of S(1-6)-NH_2 at pH ≥ 5 two main peaks were visible: a peak with the retention of S(1-6)-NH_2 and a peak with the retention of [β-Asp^3]-S(1-6)-NH_2. Treatment of the lyophilized mixtures with 1% NH_4HCO_3 did not lead to new peaks, although the relative areas of the peaks sometimes changed. After storage of S(13-18) at pH 2.5 three main peaks were visible with reverse-phase HPLC: a peak with the retention of S(13-18), a peak with the retention of [β-Asp^{15}]-S(13-18), and a peak that had almost the same

retention as S(13-18). This last mentioned peak disappeared on treatment of the lyophilized mixture with 1% NH_4HCO_3. In view of the results with S(1-6)-NH_2, we concluded that this peak was caused by [aspartoyl^{15}]-S(13-18). After storage of S(13-18) at pH $\geq$ 5 two main peaks were visible: a peak with the retention of [β-Asp^{15}]-S(13-18) and a peak with the retention of S(13-18). Treatment of the lyophilized mixtures with 1% NH_4HCO_3 did not result in other peaks.

Next, we studied the behaviour of secretin in aqueous solution. We synthesized the products which we expected, in view of the results obtained with the model peptides, to be formed from secretin: [aspartoyl^3]-secretin, [β-Asp^3]-secretin, and S(4-27). This will be described in chapter 5. These are products of an Asp^3($\alpha\rightarrow\beta$)-rearrangement and of an Asp^3-Gly bond cleavage in secretin. We succeeded in separating these peptides by RP HPLC by developing a new system, with methanol/water mixtures as the eluent and perfluoroalkanoic acids as lipophilic ion-interaction reagents (96). The peptides were found to be of low biological potency.

We studied the behaviour of secretin in aqueous solution at different pH. After storage (50 °C, 65 h) the peptide solution was analysed by amino acid analysis after enzymic hydrolysis with LAP (Table 3.4), by RP HPLC (Fig. 3.2), and by TLC.

From the results shown in Table 3.4 and Fig. 3.2 it can be concluded that at pH 2.5 [aspartoyl^3]-S is the main product, while S(4-27) is also formed. At pH $\geq$ 5 no bond cleavage was observed and the products of an Asp^3($\alpha\rightarrow\beta$)-rearrangement were secretin and [β-Asp^3]-secretin. Analysis of the product mixtures by TLC substantiated these results. The amino acid analyses of the products after hydrolysis by LAP (Table 3.4) indicate that, besides an Asp^3($\alpha\rightarrow\beta$)-rearrangement also an Asp^{15}($\alpha\rightarrow\beta$)-rearrangement might have taken place. This we did not study any further. In experiments with the model peptides we had found that the obvious approach to solve this problem, degradation of the peptide by treatment with trypsin, followed by isolation and analysis of the fragments formed (4), could lead to erroneous conclusions. Fig. 3.3 shows that the -Arg-α-Asp- bond in S(13-18), an intermediate in the tryptic hydrolysis of secretin, is hydrolysed much faster than the -Arg-β-Asp-bond in [β-Asp^{15}]-S(13-18), an intermediate in the tryptic hydrolysis of secretin if an Asp^{15}($\alpha\rightarrow\beta$)-rearrangement has occurred. So, to obtain correct results after tryptic hydrolysis, the peptides S(13-14), S(15-18), [β-Asp^{15}]-S(15-18), and [β-Asp^{15}]-S(13-18) must be determined quantitatively.

Table 3.2 Distribution of products[a] after storage of hexapeptides in aqueous solution[b]

	A; S(1-6)-NH_2						B; S(13-18)			
	Directly after storage			After lyophilization and storage in 1% NH_4HCO_3			Directly after storage		After lyophilization and storage in 1% NH_4HCO_3	
	I[c]	II[d]	III[c]	I[c]	II[d]	III[e]	I[f]	II[g]	I[f]	II[g]
pH 2.5	89	--[h]	11	60	26	14	91[j]	9	88	12
pH 5.0	79	21	--[h]	72	28	--[h]	94	6	93	7
pH 7.5	82	18	--[h]	82	18	--[h]	94	6	95	5
k	100	--[h]	--[h]	100	--[h]	--[h]	100	--[h]	96	4

a HPLC analysis on octadecylsilyl-silica with methanol/water/TFA mixtures as eluents. UV-detection at 205 nm. Only the main peaks, as mentioned in the text, are considered. The relative peak areas have been given. b Synthetic fragments of secretin, S(1-6)-NH_2 (A) and S(13-18) (B) were dissolved in 0.1 M ammonium acetate, adjusted to pH 2.5, pH 5.0, and pH 7.5, respectively (c = 0.1 mg/ml). After storage for 65 h at 50 °C, the solution was injected for analysis. After lyophilization of the remaining solution the products were dissolved in 1% NH_4HCO_3 (c = 0.2 mg/ml) and stored at ambient temperature. After 24 h, this solution was injected for analysis. c Peak with retention of S(1-6)-NH_2 (and of [aspartoyl^3]-S(1-6)-NH_2). d Peak with retention of [β-Asp^3]-S(1-6)-NH_2. e Peak with retention of S(4-6)-NH_2. f Peak with retention of S(13-18). g Peak with retention of [β-Asp^{15}]-S(13-18). h Relative peak area < 2%. j Broad peak. k Starting material.

Table 3.3 Amino acid analyses[a] of model peptides after storage in aqueous solutions[b]

	A[b]; S(1-6)-NH_2						B[b]; S(13-18)					
	His	Ser	Asp	Gly	Thr	Phe	Leu	Arg	Asp	Ser	Ala	Arg
pH 2.5	1.07	0.94	0.79	0.80	0.84	0.81	1.00	0.99	0.85	0.76	1.02	0.99
pH 5.0	1.05	0.95	0.76	0.75	0.79	0.76	1.01	0.99	0.95	0.85	1.01	0.99
pH 7.5	1.03	0.98	0.81	0.81	0.85	0.82	1.02	0.99	0.93	0.82	1.01	1.00
c	1.03	0.93	0.99	0.99	1.04	1.01	0.98	1.00	0.97	0.87	1.03	1.00

a After enzymic hydrolysis with LAP. b Synthetic fragments of secretin, S(1-6)-NH_2 (A) and S(13-18) (B) were dissolved in 0.1 M ammonium acetate, adjusted to pH 2.5, pH 5.0, and pH 7.5, respectively (c = 0.1 mg/ml).After storage for 65 h at 50 °C the solutions were lyophilized and analysed after enzymic hydrolysis. c Starting material.

Table 3.4 Amino acid analyses[a] of secretin after storage in aqueous solutions[b]

	Asp	Glu	Gly	Ala	Val	Leu	Phe	His	Arg
pH 2.5	1.54	1.08	1.70	1.02	0.98	5.98	0.97	0.93	4.06
pH 5.0	1.59	1.07	1.69	1.05	0.97	5.96	1.02	0.90	4.07
pH 7.5	1.72	1.01	1.80	1.05	0.98	5.99	0.84	0.86	4.03
c	1.97	1.12	1.92	1.01	1.00	5.95	1.09	0.92	4.06

a After enzymic hydrolysis with LAP. b Synthetic secretin was dissolved in 0.1 M ammonium acetate, adjusted to pH 2.5, pH 5.0, and pH 7.5, respectively (c = 0.5 mg/ml). After storage for 65 h at 50 °C, the solutions were lyophilized and analysed after enzymic hydrolysis. c Starting material.

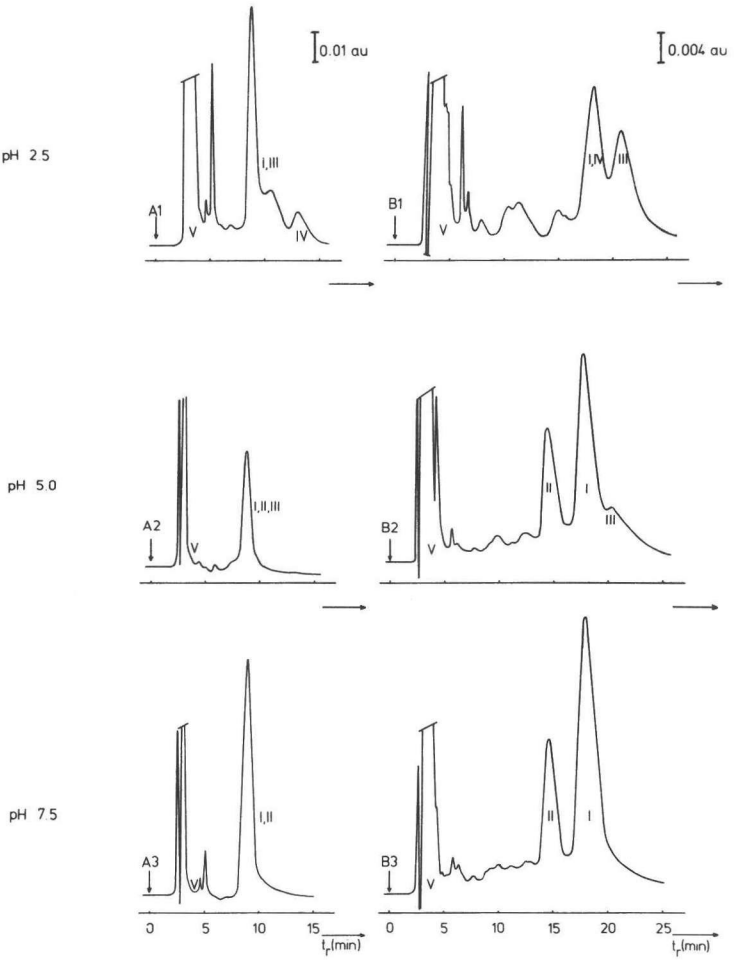

Fig. 3.2 Chromatograms of secretin after storage in aqueous solution.

I: peak with retention of secretin.

II: peak with retention of [β-Asp^3]-S.

III: peak with retention of [aspartoyl^3]-S.

IV: peak with retention of S(4-27).

V: solvent peak.

Secretin was dissolved in 0.1 M ammonium acetate, adjusted to pH 2.5 (1), pH 5.0 (2) and pH 7.5 (3), respectively (c = 0.5 mg/ml). After storage for 65 h at 50 °C 10 µl was injected for analysis (A). After lyophilization of the solutions, 50 µl 0.1 M acetic acid was added and 10 µl hereof was injected for analysis (B). Column: Polygosil C18, 10 µm (30 x 0.4 cm I.D.); eluent: methanol/water/TFA 65:35:0.1 (A), methanol/water 82:18 with 0.005 M perfluorooctanoic acid (B); UV detection at 205 nm (A) or 215 nm (B); flow rate: 1 ml/min; ambient temperature.

From the figures shown in Table 3.4 one can conclude that, even at 50 °C, no Glu(α→γ)-rearrangement or Gln deamidation has occurred to any great extent. Nor could racemization be detected. Other potential reactions, mentioned in Table 3.4 cannot be observed in this way.

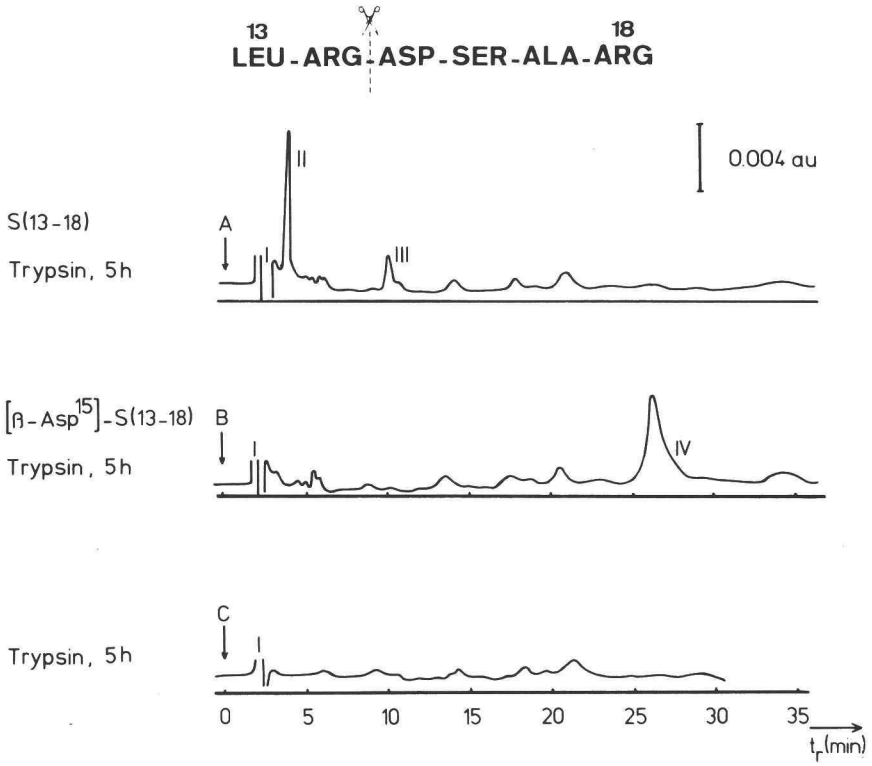

Fig. 3.3 Chromatograms of S(13-18) and [β-Asp^{15}]-S(13-18) after treatment with trypsin.

I: solvent peak.

II: peak with retention of S(15-18).

III: peak with retention of S(13-14).

IV: peak with retention of [β-Asp^{15}]-S(13-18).

S(13-18) (A) and [β-Asp^{15}]-S(13-18) (B), in 1% NH_4HCO_3 (0.2 mg/ml), were treated with trypsin (2.5 mg/mg peptide) at ambient temperature. After 5 h, the solutions were stored in boiling water for 5 min, and 10 µl of the solutions was injected for analysis. The same procedure was followed with a trypsin solution to which no peptide had been added (C).

Column: Nucleosil C18, 5 µm (15 x 0.4 cm I.D.); eluent: methanol/water/TFA 4:96:0.1; UV detection at 205 nm; flow rate: 1 ml/min; ambient temperature.

We also investigated the behaviour of secretin in aqueous solution at 25 °C after a longer period (45 days, pH 2.5, Fig. 3.4). [Aspartoyl^3]-S was the main product, while some S(4-27) was also formed. At higher pH (pH > 5) no significant Asp^3($\alpha \rightarrow \beta$)-rearrangment or Asp^3-Gly bond cleavage could be observed. In these experiments a small amount of benzene was added to prevent microbiological degradation of secretin.

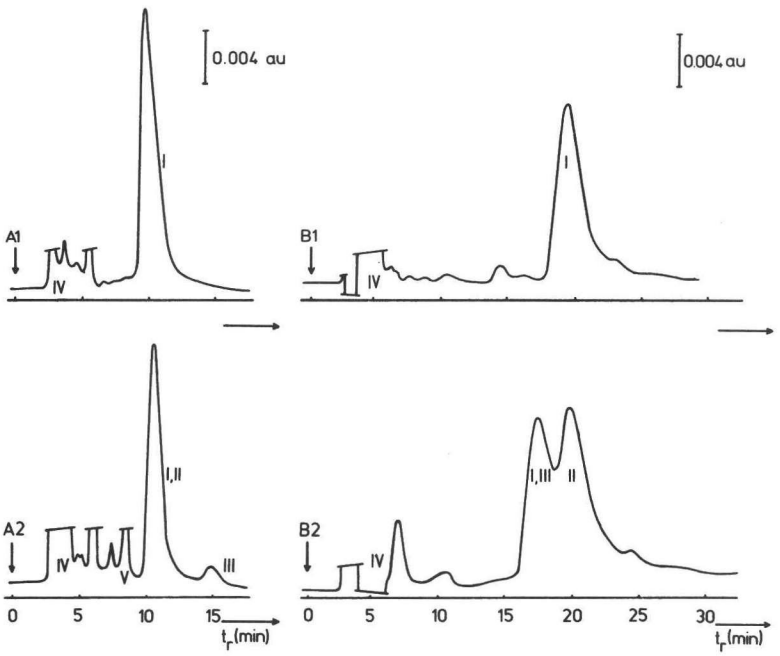

Fig. 3.4 Chromatograms of secretin before (1) and after (2) storage in aqueous solution.

I: peak with retention of secretin.

II: peak with retention of [aspartoyl^3]-S.

III: peak with retention of S(4-27).

IV: solvent peak.

V: benzene.

Secretin was dissolved in 0.1 M ammonium acetate, adjusted to pH 2.5 (c = 0.3 mg/ml). After storage at 25 °C for 45 days the solution was injected for analysis (2). 1: starting material in 0.1 M ammonium acetate. Column: Polygosil C18, 10 µm (30 x 0.4 cm I.D.); eluent: methanol/water/TFA 67:33:0.1 (A), methanol/water 83:17 with 0.005 M perfluorooctanoic acid (B); UV detection at 205 nm (A) or 215 nm (B); flow rate: 1.2 ml/min; temperature: 26 °C.

The behaviour of secretin at 25 °C was studied both in 0.1 M ammonium acetate and in 0.1 M ammonium chloride. König et al (53) have reported significant differences in stability of secretin in dilute hydrochloric acid or dilute acetic acid during the purification step. We only observed, however, small differences in reaction velocity in the two solutions studied. Consequently, in secretin several slow intramolecular reactions do occur.

Finally, we synthesized some analogues of secretin in which Gly^4 had been replaced by other amino acid residues. This will be described in the chapters 6 and 7. In these peptides the $Asp^3(\alpha\rightarrow\beta)$-rearrangement will proceed slower than in secretin. These analogues were considerable less potent in the bioassays than secretin. After our findings with secretin we did not study the stability of the analogues. These peptides were used in studies on structure-activity relationships in the secretin family, as will be described in the chapters 6 and 7.

3.5. EXPERIMENTAL PART

All synthetic peptides were prepared in this laboratory, as (will be) described (chapters 2, 4-6). The enzymic hydrolysis with LAP and the amino acid analyses were performed as described in chapter 2. Trypsin was purchased from Boehringer Mannheim, GFR.

HPLC-analyses were performed analogously to the procedure as described in chapter 2. The following columns were used: Nucleosil C18, 5 µm (15 x 0.4 cm I.D.), Polygosil C18, 10 µm (30 x 0.4 cm I.D.), or RCM-100 equipped with a Radial-Pak cartridge, type 'Delft University Special' C18, 7 µm (10 x 0.8 cm I.D.). TLC was performed on silicagel (Merck silica gel 60 F-254) in the system butanol/pyridine/acetic acid/water 60:20:6:24.

Bioassays were performed as described, with the anaesthetized rat (see chapter 2) or with the conscious dog (60) (Center for Ulcer Research and Education, Los Angeles, CA, U.S.A.), or with the anaesthetized ferret (University of Sheffield, Sheffield, U.K.) (see chapter 2).

3.6. CONCLUSIONS:

Our aim was the elucidation of the cause(s) of the decreasing biological potency of porcine secretin at the molecular level and possibly the finding of a practical remedy. From our results described here we conclude:

- The phenomenon of the loss of biological potency is difficult to demonstrate in a reproducible bioassay using various animal species.
- The loss of biological potency can be decreased or prevented by the addition of bovine serum albumin (BSA) to the secretin solution.
- Secretin possesses a strong tendency to adsorb on materials with which it comes into contact, e.g. borosilicate glass. The adsorbed peptide can be desorbed by the addition of BSA.
- A possible $Asp^3(\alpha\rightarrow\beta)$-rearrangement involves a loss of biological potency.
- In secretin an $Asp^3(\alpha\rightarrow\beta)$-rearrangement takes place slowly. At pH 2.5 [aspartoyl^3]-S is formed. In less acidic solutions a mixture of secretin and [β-Asp^3]-S is formed.
- Besides an $Asp^3(\alpha\rightarrow\beta)$-rearrangement, an Asp^3-Gly peptide chain cleavage occurs in secretin.
- Besides an $Asp^3(\alpha\rightarrow\beta)$-rearrangement, possibly an $Asp^{15}(\alpha\rightarrow\beta)$-rearrangement might occur in secretin.
- Several analogues of secretin, in which Gly^4 has been replaced by other amino acid residues, are considerably less potent than secretin in the bioassays used.

Consequently, the rapid loss of potency in vitro appears to be caused by a reversible physical adsorption on glass or synthetic materials. This can be prevented by the addition of BSA.

Some chemical reactions, like the $Asp^3(\alpha\rightarrow\beta)$-rearrangement, splitting of the peptide chain, and possibly the $Asp^{15}(\alpha\rightarrow\beta)$-rearrangement proceed only slowly. This may explain a slow loss of biological potency in vitro. $Glu(\alpha\rightarrow\gamma)$-rearrangements, deamidation of Gln, and racemization could not be detected. Whether these reactions have any significance in the in vivo inactivation of secretin remains to be answered.

In this chapter no direct correlation between biological potency and chemical characteristics of secretin can be described. Only a direct comparison of a sufficient large number of data for various secretin samples permits quantitative conclusions about the stability of secretin.

4 SYNTHESIS AND SOME PROPERTIES OF MODEL PEPTIDES

4.1 INTRODUCTION

The $Asp^3(\alpha\rightarrow\beta)$-rearrangement was generally thought to be responsible for the inactivation of secretin. In secretin two Asp residues occur (Fig. 4.1): Asp^3, followed by Gly^4, and Asp^{15}, followed by Ser^{16}.

His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-
1 2 3 4 5 6 7 8 9

-Leu-Ser-Arg-Leu-Arg-Asp-Ser-Ala-Arg-
10 11 12 13 14 15 16 17 18

-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH_2
19 20 21 22 23 24 25 26 27

Fig. 4.1 The sequence of secretin and model peptides (underlined)

It is known, from investigations with small protected peptides, that the occurrence of the $Asp(\alpha\rightarrow\beta)$-rearrangement is strongly dependent on the amino acid residue that follows the Asp residue (47, 49) in the sequence. The rearrangement occurs relatively fast if this is a Ser, Thr, or Gly residue. In the sequences -Asp-Gly- the $Asp(\alpha\rightarrow\beta)$-rearrangement is not seriously hindered by a sterically bulky side-chain of the following Gly residue. In the sequences -Asp-Ser- or -Asp-Thr- the $Asp(\alpha\rightarrow\beta)$-rearrangement might be catalyzed by the hydroxyl group in the side-chain of the following Ser or Thr residue (Fig. 4.2). We decided not only to study the behaviour of an Asp-Gly sequence, but also of an Asp-Ser sequence.

We chose $S(1-6)-NH_2$ and S(13-18) as model peptides. In these peptides the direct environment of the Asp-residues is similar to that in secretin, so far as possible in model peptides (Fig. 4.1). We synthesized $S(1-6)-NH_2$, $[\beta\text{-}Asp^3]\text{-}S(1-6)-NH_2$, $[aspartoyl^3]\text{-}S(1-6)-NH_2$, S(13-18), and $[\beta\text{-}Asp^{15}]$-S(13-18).

During the experiments with the model peptides we met some unforeseen phenomena, viz. a peptide chain cleavage and a slow hydrolysis of the β-Asp-Ser- peptide bond with trypsin. In order to be able to study these phenomena more extensively we synthesized S(4-6)-NH_2, S(13-14), and S(15-18).

In this chapter the preparation and some properties of the model peptides are described. The peptide S(1-6)-NH_2 has also been synthesized by Ondetti et al. (46), as a model peptide in order to study problems in the synthesis of secretin.

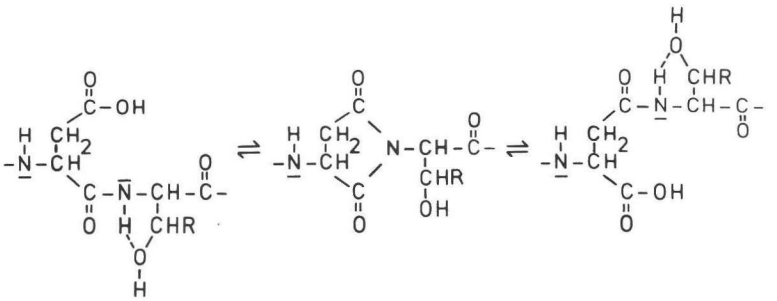

Fig. 4.2 The Asp(α→β)-rearrangement in peptides
R=H: Asp-Ser; R=CH_3: Asp-Thr

4.2 EXPERIMENTS AND RESULTS

Protected S(1-6)-NH_2 was synthesized as shown in Fig. 4.3. The N_{im}-Tos protection was removed by treatment with pyridine.HCl (56) and the product was purified by gel permeation chromatography. Deprotection by acidolysis with TFA yielded a practically pure S(1-6)-NH_2 (HPLC, TLC, amino acid analysis (Table 4.1)).

Protected [aspartoyl^3]-S(1-6)-NH_2 was synthesized as shown in Fig. 4.4. Deprotection by acidolysis with HF/anisole followed by purification by HPLC yielded a practically pure [aspartoyl^3]-S(1-6)-NH_2 (HPLC, TLC, amino acid analysis (Table 4.1)).

Protected [β-Asp^3]-S(1-6)-NH_2 was synthesized as shown in Fig. 4.5. After treatment with pyridine.HCl, gel permeation chromatography and treatment with TFA a practically pure [β-Asp^3]-S(1-6)-NH_2 was obtained (HPLC, TLC, amino acid analysis (Table 4.1)).

Protected S(13-18)-OCH_3 was synthesized as shown in Fig. 4.6.

Deprotection by acidolysis with HF/anisole yielded a product which did not contain any significant [aspartoyl^{15}]-S(13-18), according to amino acid

analysis after enzymic hydrolysis (LAP). During storage of this crude S(13-18)-OCH_3 in 0.1 M ammonium acetate, adjusted to pH 7.5, for 250 h at 50 °C a reaction mixture was obtained with S(13-18) and [β-Asp^{15}]-S(13-18) as the main products. These products were purified by preparative HPLC, which yielded practically pure S(13-18) and [β-Asp^{15}]-S(13-18) (HPLC, TLC, amino acid analysis (Table 4.1)).

Protected S(15-18) was obtained starting with protected S(16-18)-OCH_3, an intermediate in the synthesis of S(13-18) (Fig. 4.6). After base catalyzed hydrolysis and treatment with TFA, Boc-Asp(OBzl)-ONp was coupled. After purification by gel permeation chromatography the protecting groups were removed by acidolysis with TFA, followed by catalytic hydrogenolysis. After purification by ion-exchange chromatography, a practically pure product was obtained (HPLC, TLC, amino acid analysis (Table 4.1)).

Protected S(13-14)-OCH_3 was obtained after coupling Boc-Leu to Arg(NO_2)-OCH_3 by the mixed anhydride method. After base catalyzed hydrolysis of the methyl ester, treatment with TFA, catalytic hydrogenolysis, and purification by ion-exchange chromatography a practically pure product was obtained (HPLC, TLC, amino acid analysis (Table 4.1)).

The peptide S(4-6)-NH_2 was obtained as an intermediate in the synthesis of S(1-6)-NH_2. This peptide was practically pure according to amino acid analysis (Table 4.1), HPLC and TLC.

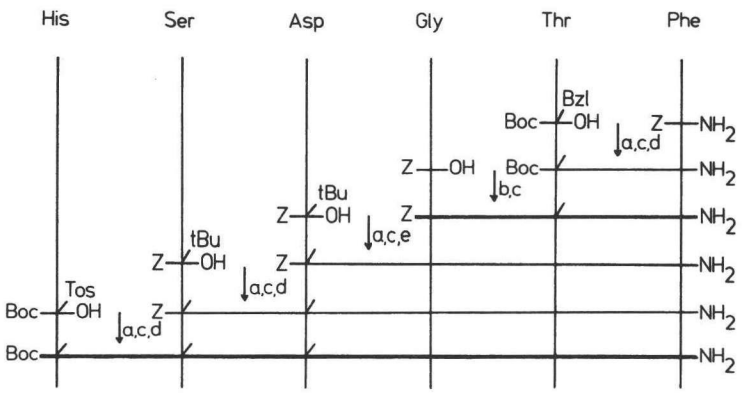

Fig. 4.3 The synthesis of protected S(1-6)-NH_2

a Deprotection by catalytic hydrogenolysis at ambient temperature.

b Deprotection by acidolysis with TFA (30 min, ambient temperature).

c Coupling by the excess mixed anhydride method.

d Reaction conditions for hydrogenolysis: 80% aqueous acetic acid; 4-8 h.

e Reaction conditions for hydrogenolysis: 80% aqueous acetic acid; 24 h.

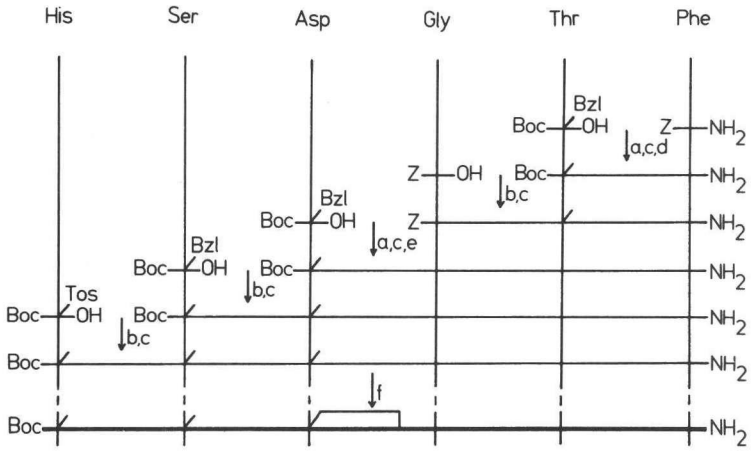

Fig. 4.4 The synthesis of protected [aspartoyl^3]-S(1-6)-NH_2

a-e See Fig. 4.3.

f See experimental part (p. 52).

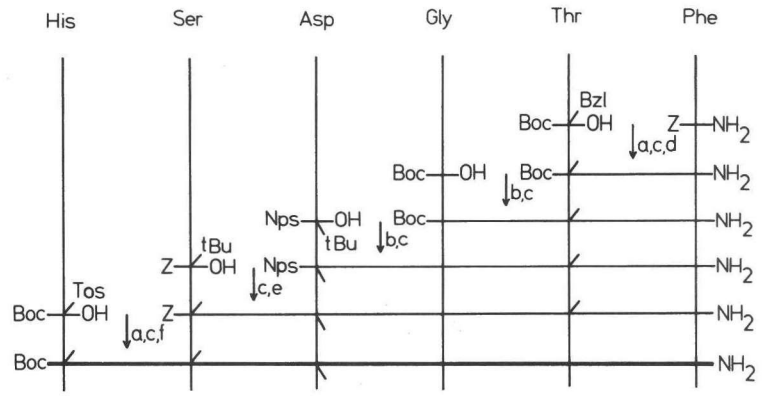

Fig. 4.5 The synthesis of protected [β-Asp^3]-S(1-6)-NH_2

a-d See Fig. 4.3.

e Deprotection by acidolysis by HCl in methanol.

f 80% aqueous acetic acid; 30 h.

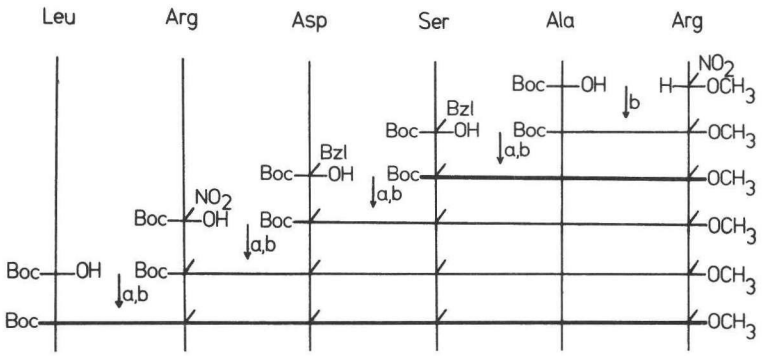

Fig. 4.6 The synthesis of protected S(13-18)-OCH_3

a Deprotection by acidolysis with TFA (30 min, ambient temperature).

b Coupling by the excess mixed anhydride method.

Experimental data of the syntheses are summarized in Table 4.3.

Some electrophoretic data of S(1-6)-NH_2, [β-Asp^3]-S(1-6)-NH_2, and [$aspartoyl^3$]-S(1-6)-NH_2 are presented in Table 4.2.

Chromatographic data of the synthetic peptides are given in Table 4.4.

The stability of [$aspartoyl^3$]-S(1-6)-NH_2, under the circumstances that occur during e.g. enzymic hydrolysis with trypsin, was studied with the aid of HPLC (Table 4.5).

Table 4.1 Amino acid analyses of model peptides

		His	Ser	Asp	Gly	Thr	Phe
S(1-6)-NH_2	a	1.01	0.90	0.99	1.02	0.95	0.98
	b	1.01	0.96	0.99	0.98	1.04	1.01
[β-Asp^3]-S(1-6)-NH_2	a	1.02	0.85	0.94	1.03	0.97	1.00
	b	0.99	0.98	0.08	0.08	0.12	1.01
[$Aspartoyl^3$]-S(1-6)-NH_2	a	1.00	0.87	1.01	1.00	0.93	0.98
	b	1.14	0.86	0.13	0.13	0.15	0.22
S(4-6)-NH_2	a				1.00	0.96	1.00
	b				0.99	1.01	1.00

		Leu	Arg+ Orn	Asp	Ser	Ala
S(13-18)	a	0.96	1.97	1.05	0.87	0.92
	b	1.06	1.92	1.01	1.01	1.01
[β-Asp^{15}]-S(13-18)	a	1.05	1.91	1.05	0.82	0.94
	b	1.00	1.31	0.04	0.03	0.48
S(13-14)	a	1.01	0.99			
	b	1.01	0.99			
S(15-18)	a		1.02	1.02	0.81	0.95
	b		1.01	1.03	0.97	1.00

a Acidic hydrolysis (6 M HCl, 24 h, 110 °C).

b Enzymic hydrolysis (LAP, 24 h, 37 °C).

Table 4.2 Paper electrophoresis of S(1-6)-NH_2 and analogues

	S(1-6)-NH_2	[β-Asp^3]-S(1-6)-NH_2	[$Aspartoyl^3$]-S(1-6)-NH_2
R_f [a,b]	0.66	0.62	0.58
R_f [a,c]	0.29	0.27	0.80

a Compared with the behaviour of His: $R_{f,His} = 1$.

b Acetic acid/formic acid/water 15:5:100; pH 1.9.

c Pyridine/acetic acid/water 10:1:90; pH 6.5.

Table 4.3 Experimental data of the syntheses of model peptides

Protected peptides	Deprotection			Purification		
	Method	Starting material (mg)	Yield (mg)	Method	Starting material (mg)	Yield (mg)
Boc-His(Tos)-Ser(tBu)-Asp(OtBu)-Gly-Thr-Phe-NH_2	pyridine.HCl;TFA	150	100			
Boc-His(Tos)-Ser(tBu)-Asp-Gly-Thr-Phe-NH_2	HF/anisole	250	191	HPLC[a]	5.5	3.5
Boc-His(Tos)-Ser(tBu)-Asp(α-OtBu)-Gly-Thr-Phe-NH_2	pyridine.HCl;TFA	22	10			
Boc-Leu-Arg(NO_2)-Asp(OBzl)-Ser(Bzl)-Ala-Arg(NO_2)-OCH_3	HF/anisole	364	230	HPLC[b]	30	7[c] 8.5[d]
Boc-Asp(OBzl)-Ser(Bzl)-Ala-Arg(NO_2)-OH	TFA;H_2,Pd/C[e]	500	550	Ion-exchange chromatography[f]	65	31
Boc-Leu-Arg(NO_2)-OCH_3	NaOH;TFA;H_2,Pd/C[e]	446	432	Ion-exchange chromatography[g]	80	45

a Polygosil C18, 10 µm (20 x 0.9 cm I.D.); methanol/water/TFA 15:85:0.5; ambient temperature.

b S(13-18)-OCH_3 was dissolved in 0.1 M ammonium acetate, adjusted to pH 7.5 (c = 10 mg/ml). After storage for 250 h at 50 °C, the solution was lyophilized. The resulting mixture was chromatographed (LiChrosorb RP-18, 10 µm (20 x 0.9 cm I.D.); methanol/water/TFA 14:86:0.5; ambient temperature).

c [β-Asp^{15}]-S(13-18).

d S(13-18).

e 80% aqueous acetic acid.

f DEAE-Sephadex C-25; 0.01 M NH_4HCO_3 (pH 6.0).

g SP-Sephadex C-25; 0.01 M NH_4HCO_3 (pH 5.7).

Table 4.4 Chromatographic data of model peptides

	R_f [a,b]	R_f [a,c]	R_f [a,d]	k' [e,f]	k' [e,g]
S(1-6)-NH_2	0.24		0.12	6.3	
[β-Asp^3]-S(1-6)-NH_2	0.19		0.09	5.4	
[$Aspartoyl^3$]-S(1-6)-NH_2	0.17		0.18	6.3	
S(4-6)-NH_2		0.49	0.40	3.9	
S(13-18)		0.05		3.2	
[β-Asp^{15}]-S(13-18)		0.04		2.2	
S(13-14)		0.22	0.18		6.0
S(15-18)		0.06	0.03		1.0

a TLC; silicagel.

b n-Amylalcohol/pyridine/water 7:7:6.

c Butanol/pyridine/acetic acid/water 45:20:6:24.

d Butanol/pyridine/acetic acid/water 60:20:6:24.

e HPLC; Polygosil C18, 10 µm (30 x 0.4 cm I.D.), 30 °C.

f Methanol/water/TFA 15:85:0.25.

g Methanol/water/TFA 5:95:0.25.

Table 4.5 The stability of [$aspartoyl^3$]-S(1-6)-NH_2

Solvent	Reaction time[a]	Product distribution[b] I	II
0.1 M ammonium acetate, pH 7.5	72 h	33	67
pH 5.0	72 h	94	6
pH 2.5	72 h	100	--[c]
1% NH_4HCO_3; pH 7.9	10 min	75	25
	40 min	32	68
	85 min	21	79
	150 min	22	78

a [$Aspartoyl^3$]-S(1-6)-NH_2 was dissolved (c = 0.15 mg/ml) and after storage at ambient temperature the reaction mixture was analysed with the aid of HPLC.

b The relative peak area has been given (UV detection at 205 nm).
I: S(1-6)-NH_2 and [$aspartoyl^3$]-S(1-6)-NH_2; II: [β-Asp^3]-S(1-6)-NH_2.

c Relative peak area < 2%.

4.3 EXPERIMENTAL PART

Reagents, solvents, and amino acid derivatives were purchased, except Nps-Asp(α-OtBu).DCHA, Arg(NO_2)-OCH_3.HCl, Z-Val-NH_2, and Boc-Asp(OBzl)-ONp, which were prepared in this laboratory.

For the technique of coupling of protected amino acids by the REMA method we refer to chapter 2. The protected peptides were isolated as described in chapter 2 or by extraction of the reaction mixture with ethyl acetate, washing of the organic layer with water, drying of the organic layer ($MgSO_4$), and evaporation, after filtration, of the organic layer in vacuo. If the excess of the protected amino acid could not be removed satisfactorily by extraction the product mixture was chromatographed on a column, filled with aluminium oxide (Merck, Darmstadt, GFR; activity I (20 x 2.5cm I.D.) with chloroform/methanol mixtures as the eluent, after which the main fractions were collected and evaporated in vacuo. The Boc-group was removed by acidolysis in TFA or TFA/CH_2Cl_2 (1:1), analogously to the procedure described in chapter 2. The Z-group was removed by catalytic hydrogenolysis, analogously to the procedure as will be described for the deprotection of Z-Ser(tBu)-Asp(OtBu)-Gly-Thr-Phe-NH_2 (p. 52). The removal of side-chain protecting groups by catalytic hydrogenolysis was performed analogously to the removal of the Z-group. The Tos-group was removed by treatment with pyridine.HCl, analogously to the procedure as will be described for the deprotection of Boc-His(Tos)-Ser(tBu)-Asp(OtBu)-Gly-Thr-Phe-NH_2 (p. 52). The hydrolysis of the methylester was performed analogously to the procedure as will be described for the hydrolysis of Boc-Ser(Bzl)-Ala-Arg(NO_2)-OCH_3 (p. 53). The removal of protecting groups by acidolysis with the aid of HF/anisole, the purification of peptides by ion-exchange chromatography or HPLC, and the amino acid analysis were performed analogously to the procedures as described in chapter 2. The acidic and enzymic hydrolyses of peptides were performed as described in chapter 2. RP-HPLC was performed on octadecylsilyl-silica with methanol/water/TFA mixtures as the eluents. The final products were analysed on Nucleosil C18, 5 μm (15 x 0.4 cm I.D.) or Polygosil C18, 10 μm (30 x 0.4 cm I.D.) (UV-detection at 205 nm). TLC was performed as described in chapter 2. The final products were analysed in the TLC systems mentioned in Table 4.4. Electrophoresis was performed on paper (Whatman nr I, 1200 V; 40 mA, -5 °C). The spots were made visible by spraying with ninhydrin, followed by heating for about 15 min (110 °C).

THE SYNTHESIS OF PROTECTED [ASPARTOYL^3]-S(1-6)-NH_2

Boc-His(Tos)-Ser(Bzl)-Asp(OBzl)-Gly-Thr-Phe-NH_2 (0.28 g, 0.26 mmol) was dissolved in 20 ml of methanol. NMM (30 µl, 0.27 mmol) was added. The reaction was monitored with the aid of HPLC. After storage for 70 min at ambient temperature the mixture was added dropwise to 80 ml of ice water. The precipitate formed thus was filtered off, washed with water and dried in vacuo.
Yield: 0.25 g (0.25 mmol; 97%).

THE DEPROTECTION OF Nps-Asp(α-OtBu)-Gly-Thr(Bzl)-Phe-NH_2

Nps-Asp(α-OtBu)-Gly-Thr(Bzl)-Phe-NH_2 (150 mg; 0.20 mmol) was dissolved in 25 ml of methanol. A solution of 0.05 M HCl in methanol was added dropwise to the peptide solution at ambient temperature, so that pH > 4. The reaction was monitored by TLC. After completion of the reaction the reaction mixture was concentrated in vacuo, triturated with ether, the precipitate was filtered off and dried in vacuo.

The residue was coupled to Z-Ser(tBu) by the excess mixed anhydride method.
Yield: 142 mg (0.16 mmol; 81%).

THE DEPROTECTION OF Z-Ser(tBu)-Asp(OtBu)-Gly-Thr-Phe-NH_2

Z-Ser(tBu)-Asp(OtBu)-Gly-Thr-Phe-NH_2 (0.38 g, 0.49 mmol) was dissolved in 25 ml of 80% aqueous acetic acid. To this solution 100 mg of the catalyst (10% Pd on charcoal; Drijfhout, Amsterdam, Holland) was added. The mixture was stirred under a hydrogen atmosphere for 8 h. The catalyst was filtered off, washed with acetic acid, and the filtrate was lyophilized.

The residue was coupled to Boc-His(Tos) by the excess mixed anhydride method.
Yield: 0.33 g (0.32 mmol, 65%).

THE DEPROTECTION OF PROTECTED S(1-6)-NH_2

Protected S(1-6)-NH_2 (0.15 g; 0.15 mmol) was dissolved in 3 ml of DMF. Pyridine.HCl (174 mg; 1.5 mmol) was added to this solution. After storage for 5 h the solution was chromatographed (Sephadex LH-20; DMF). The fractions containing the main product were collected, concentrated in vacuo, and lyophilized from acetic acid.
Yield: 0.12 g.

This product was dissolved in 12 ml of TFA. After storage for 4 h, 100 ml of acetic acid was added and the solution was lyophilized.
Yield: 0.10 g.

THE SYNTHESIS OF Boc-Asp(OBzl)-Ser(Bzl)-Ala-Arg(NO_2)

Boc-Ser(Bzl)-Ala-Arg(NO_2)-OCH_3 (2.1 g, 3.6 mmol) was dissolved in 30 ml of dioxan and 3 ml of water. To this solution 4 ml of 2 M NaOH was added. After stirring for 30 min at ambient temperature the pH of the solution was adjusted to pH ~ 4 with 2 M HCl. After the addition of 100 ml of water the solution was extracted with ethyl acetate. The organic layer was washed with water, dried ($MgSO_4$), and, after filtration, evaporated in vacuo.

The resulting oil was dissolved in 30 ml of TFA. After storage for 30 min the solution was evaporated, the peptide material was triturated with ether, filtrated, washed with ether and dried in vacuo.

The resulting oil was dissolved in 5 ml of DMF. The pH of the solution was adjusted to pH 7 (with NMM) and NMM (0.4 ml, 3.6 mmol) was added. Boc-Asp(OBzl)-ONp (2.0 g, 4.5 mmol) was added. After stirring for 48 h at ambient temperature the reaction mixture was concentrated in vacuo. The resulting oil was chromatographed in seven portions (Sephadex LH-20 (90 x 2.5 cm I.D.), methanol). The main fractions were collected, evaporated in vacuo, and lyophilized from acetic acid.
Yield: 2.4 g (3.1 mmol, 85%).

4.4 CONCLUSIONS

The peptides S(1-6)-NH_2, [aspartoyl^3]-S(1-6)-NH_2, [β-Asp^3]-S(1-6)-NH_2, S(13-18), and [β-Asp^{15}]-S(13-18) have been synthesized.

It was possible to separate S(1-6)-NH_2 and [β-Asp^3]-S(1-6)-NH_2 by reverse-phase HPLC in several systems. It was not possible to separate S(1-6)-NH_2 and [aspartoyl^3]-S(1-6)-NH_2 in any of these systems (96). In weakly basic solution, however, a mixture of S(1-6)-NH_2 and [β-Asp^3]-S(1-6)-NH_2 is rapidly formed from [aspartoyl^3]-S(1-6)-NH_2. This makes it possible to estimate the amount of [aspartoyl^3]-S(1-6)-NH_2 that is present together with S(1-6)-NH_2. A possible Asp^3(α→β)-rearrangement can be followed thus by HPLC-analysis.

The peptides S(13-18) and [β-Asp^{15}]-S(13-18) could be separated by reverse-phase HPLC in several systems. [Aspartoyl^{15}]-S(13-18) has not been synthesized. In view of the results obtained with S(1-6)-NH_2 and analogues it is reasonable to expect that the amount of [aspartoyl^{15}]-S(13-18) can be estimated in the same way as described for [aspartoyl^{3}]-S(1-6)-NH_2.

The peptides S(4-6)-NH_2, S(13-14), and S(15-18) have been synthesized. These peptides were used to study some phenomena observed during experiments with model peptides, as described in chapter 3.

5. SYNTHESIS AND SOME PROPERTIES OF [ASPARTOYL^3]-SECRETIN, [β-ASP^3]-SECRETIN, AND SECRETIN(4-27)

5.1 INTRODUCTION

Several workers have reported that secretin rapidly lost biological potency on storage, especially in aqueous solution. This phenomenon was studied in our laboratory, as described in chapter 3. We found that in secretin an Asp^3(α→β)-rearrangement (Fig. 2.3) and an Asp^3-Gly bond cleavage take place slowly in aqueous solution. In order to study these reactions we needed potential reaction products. We wish to report here the preparation, the biological and the immunological potency, and some chromatographic properties of [aspartoyl^3]-S, [β-Asp^3]-S, and S(4-27). These peptides have been synthesized by several groups, each with a different strategy, but insufficient data were given to be able to study intramolecular reactions in secretin (9, 16, 37).

5.2 EXPERIMENTS AND RESULTS

The syntheses of side-chain protected S(4-27) and fully protected [β-Asp^3]-S were performed analogously to the synthesis of protected secretin, as shown in Fig. 2.1a. Deprotection by catalytic hydrogenolysis and successive purification by ion-exchange chromatography and by preparative reverse-phase HPLC yielded practically pure products (TLC, HPLC, amino acid analyses (Table 5.2)).

[Aspartoyl^3]-S was synthesized as shown in Table 5.1. Following this strategy, a practically pure product was obtained (TLC, HPLC, amino acid analysis (Table 5.2)).

Table 5.1 Strategy for the synthesis of [aspartoyl^3]-secretin

1. Synthesis of protected secretin, as shown in Fig. 2.1b
2. Formation of protected [aspartoyl^3]-S
3. Preparative HPLC purification of protected [aspartoyl^3]-S
4. Removal of protecting groups with the aid of HF/anisole
5. Preparative HPLC purification of [aspartoyl^3]-S

Table 5.2 Amino acid analyses of analogues of secretin (S)

	[Aspartoyl^3]-S					[β-Asp^3]-S				S(4-27)			
	a d	b d	c d	c e	Theory	a d	c d	c e	Theory	a d	c d	c e	Theory
Asp	1.69	1.82	2.05	1.01	2 (1.20^{f,g})	1.87	2.00	1.00	2 (1^g)	0.95	1.04	0.97	1
Thr	1.15	1.85	1.93		2	1.46	1.85		2	1.41	1.74		2
Ser	1.66	3.17	3.50		4	2.91	3.32		4	2.34	2.56		3
Glu	2.92	2.96	3.01	1.10	3 (1^h)	2.92	2.93	0.98	3 (1^h)	3.01	3.14	1.03	3 (1^h)
Gly	1.82	1.99	2.05	1.12	2 (1.20^{f,g})	1.71	2.05	1.02	2 (1^g)	1.92	2.12	1.89	2
Ala	1.12	1.08	1.07	1.03	1	1.05	1.08	1.05	1	1.23	1.12	1.03	1
Val	1.00	1.02	1.00	1.14	1	1.00	0.97	1.03	1	1.08	1.04	1.01	1
Leu	6.05	6.10	5.96	5.82	6	6.17	6.10	6.06	6	5.96	5.62	6.12	6
Phe	0.81	0.96	0.98	0.87	1	0.81	0.98	0.91	1	0.76	0.94	0.91	1
His	0.79	0.77	0.92	0.97	1	0.76	0.95	0.98	1				
Arg+Orn	3.91	3.84	3.96	3.92	4	3.86	3.95	3.88	4	3.77	3.98	4.04	4

a Crude protected peptide.

b Purified protected peptide.

c Purified peptide.

d Acidic hydrolysis (6 M HCl, 24 h, 110 °C). Gln was determined as Glu.

e Enzymic hydrolysis (LAP, 24 h, 37 °C). Thr, Ser, and Gln could not be determined.

f Estimates after enzymic hydrolysis.

g The β-Asp peptide bond was not hydrolysed with LAP.

h Glu = 1; Gln = 2.

Table 5.3 Experimental data of the synthesis of analogues of secretin (S)

Peptide	Deprotection			Purification			
	Method	Starting material (mg)	Yield (mg)	Starting material (mg)[a]	Yield (mg)[a]	Starting material (mg)[b]	Yield (mg)[b,c]
S(4-27)	H_2, Pd/C	43	26	26	6	6[d]	0.5
[β-Asp^3]-S	H_2, Pd/C	110	90	90	18	4[e]	1.5
[Aspartoyl^3]-S	HF/anisole	33	26			7[e]	2.5

a Ion-exchange chromatography (SP-Sephadex C-25; 0.02 M NH_4HCO_3 (pH 6.1), followed by 0.05 M NH_4HCO_3 (pH 6.5)).

b HPLC; methanol/water/TFA 69:31:0.5; ambient temperature.

c Corrected for peptide content. All the other figures have not been corrected.

d Polygosil C18, 10 µm (20 x 0.8 cm I.D.).

e LiChrosorb RP-18, 10 µm (20 x 0.8 cm I.D.).

Table 5.4 Chromatographic data of secretin (S) and analogues

	S	S(4-27)	[β-Asp^3]-S	[Aspartoyl^3]-S
R_f [a]	0.12	0.18	0.11	0.15
k' [b]	2.2	3.2	2.2	2.2

a TLC; silicagel; butanol/pyridine/acetic acid/water 60:20:6:24.

b HPLC; Nucleosil C18, 7 µm (15 x 0.4 cm I.D.); methanol/water 65:35 with 0.01 M TFA; ambient temperature.

Experimental data of the syntheses are given in Table 5.3.

Some chromatographic data of the peptides and secretin, in the systems used in order to check the purity, are given in Table 5.4. Electrophoretic data of the peptides and secretin are given in Table 5.5.

Table 5.5 Paper electrophoresis of secretin (S) and analogues

	S	[β-Asp^3]-S	[Aspartoyl^3]-S
R_f [a,b]	0.61	0.61	0.64
R_f [a,c]	0.40	0.40	0.52

a Compared with the behaviour of His: $R_{f,His} = 1$.
b Acetic acid/formic acid/water 15:5:100; pH 1.9.
c Pyridine/acetic acid/water 10:1:90; pH 6.5.

The analogues were assayed for their potency to stimulate the exocrine pancreatic secretion in the anaesthetized ferret, by Professor T. Scratcherd, Sheffield, England, and in the anaesthetized rat, by Dr. T.E. Solomon, Los Angeles, CA, USA (Table 5.6).

Table 5.6 Biological potency of analogues of secretin (S)

Peptide	Biological potency[a] (%)	animal	n[b]	m[c]
S(4-27)	0.1	ferret[d]	1	1
	<2, >0.04	rat[e]	1	5
[β-Asp^3]-S	0.6	ferret[d]	2	2
	0.4	rat[f]	1	3
[Aspartoyl^3]-S	2.5	ferret[d]	3	2
	2.7	rat[f]	1	6

a The analogues were compared with pure secretin, the potency of which was assumed to be 100%.
b Number of assays per dose in one animal.
c Number of animals used.
d Only one dose was administered.
e Two doses were administered.
f Three doses were administerd.

The immunological potency was determined by Dr. T.M. Chang, Rochester, NY, USA. Fig. 5.1 shows the displacement of [^{125}I-His^{1}]-secretin from secretin antibody by natural secretin and the analogues.

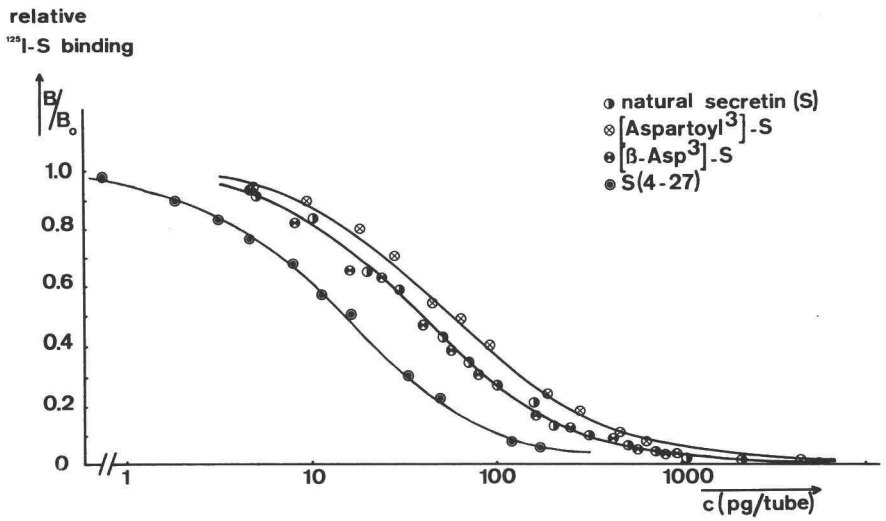

Fig. 5.1 The displacement of [^{125}I-His^{1}]-secretin from secretin antibody by natural secretin and synthetic secretin analogues (see chapter 2.2.7).

HPLC analysis of [aspartoyl^{3}]-S and [β-Asp^{3}]-S in a system developed here showed that these peptides did not contain appreciable amounts of other products that would originate from an Asp^{3}(α→β)-rearrangement in secretin. Table 5.7 shows k'-values of the peptides and secretin in such a system.

Table 5.7. Chromatographic data of secretin (S) and analogues

	S	S(4-27)	[β-Asp^{3}]-S	[Aspartoyl^{3}]-S
k' [a]	7.9	7.6	6.5	10.0

a HPLC; Nucleosil C18, 7 µm (15 x 0.4 cm I.D.); methanol/water 79:21 with 0.005 M perfluorooctanoic acid; ambient temperature.

5.3 EXPERIMENTAL PART

Reagents, solvents, and amino acid derivatives were purchased, except for Boc-Asp(α-OBzl).DCHA, which was prepared in our laboratory.

For the technique of coupling of protected amino acids by the REMA method, for the removal of protecting groups, and for the analysis and purification of protected and unprotected peptides we refer to chapter 2. Electrophoresis was performed as described in chapter 4.

Bioassays were performed by Prof. T. Scratcherd, Department of Physiology, University of Sheffield, Sheffield, England, with the anaesthetized ferret, and by Dr. T.E. Solomon, Center for Ulcer Research and Education (CURE), Veterans Administration, Los Angeles, CA, USA, with the anaesthetized rat. Details are given in chapter 2. Radioimmunoassays were performed by Dr. T.M. Chang, The Genesee Hospital, The Isaac Gordon Center for Digestive Diseases and Nutrition, Rochester, NY, USA. [Aspartoyl^3]-S and [β-Asp^3]-S were dissolved and assayed in a pH 5.0 buffer instead of the pH 7.0 buffer, usually used (57). Otherwise [aspartoyl^3]-S could not be assayed, because of the instability of the peptide at pH 7.0. The results obtained with different analogues (obtained at different dates) have been corrected so that the immunological properties of the analogues can be compared as shown in the figure.

THE SYNTHESIS OF PROTECTED [ASPARTOYL^3]-SECRETIN

Protected secretin (120 mg, 28 μmol, synthesized as described in chapter 2), was dissolved in dimethylformamide (5 ml), to which N-methylmorpholine (5.5 μl, 1.8 eq) was added. The formation of protected [aspartoyl^3]-S was monitored by reverse-phase HPLC (see Fig. 2.4). After stirring for 105 minutes at 33 °C, acetic acid (100 μl) was added in order to stop the reaction (pH ~ 4). To this solution a mixture of methanol/water/acetic acid (50:11:1, 10.8 ml) was added with vigorous stirring. After filtration of the cloudy solution, the clear solution was chromatographed (LiChrosorb RP-18, 10 μm (20 x 0.8 cm I.D.); methanol/DMF/water/acetic acid 500:300:110:25; injection: 17 x 0.75 ml). The main fractions, according to UV detection at 260 nm, were collected, evaporated in vacuo, and lyophilized from acetic acid.
Yield: 35 mg (41%). Residue after filtration: 15 mg. Amino acid analyses after acidic hydrolysis (before and after purification): see Table 5.2. The product obtained was practically pure on HPLC (Nucleosil C18, 7 μm; methanol/water/acetic acid 91:8:1, UV detection at 260 nm).

5.4 CONCLUSIONS

The peptides S(4-27), [β-Asp^3]-S, and [aspartoyl^3]-S were synthesized. The syntheses of S(4-27) and [β-Asp^3]-S gave no problems. The synthesis of [aspartoyl^3]-S, however, could only be performed after the development of suitable HPLC systems (Table 5.1), partly discussed in chapter 2. Especially the preparative HPLC-purification of protected [aspartoyl^3]-S was found to be a great improvement in the synthesis. This made it possible to obtain a practically pure peptide free of secretin and of [β-Asp^3]-S. These two compounds are quite easily formed from [aspartoyl^3]-S in nearly neutral solutions, according to Jaeger et al. (16, 64) and our observations.

The biological potency of all of the analogues described above was greatly decreased, as determined with the rat and ferret, in comparison with secretin (Table 5.6). Consequently, the changes made in the N-terminal sequence of secretin affect the biological potency of the hormone. It should be remarked that the low potency observed for [β-Asp^3]-S and [aspartoyl^3]-S might be caused by secretin formed during the synthesis or while performing bioassay experiments. The figures obtained are in accordance with reported data. An Asp^3(α→β)-rearrangement or an Asp^3-Gly bond cleavage in secretin is therefore indeed accompanied by a loss of biological potency. The immunological properties of secretin and the analogues described here were similar. The N-terminal tripeptide of secretin is therefore no (part of an) antigenic determinant, for the antibody used.

The peptides secretin, [β-Asp^3]-S, [aspartoyl^3]-S, and S(4-27) can be separated by reverse-phase HPLC. Consequently, it is possible now to study the Asp^3(α→β)-rearrangement and the Asp^3-Gly peptide bond cleavage in secretin directly in quite simple systems.

6. SYNTHESIS AND SOME PROPERTIES OF [ALA^4]-SECRETIN AND [SAR^4]-SECRETIN

6.1 INTRODUCTION

It has been reported several times that secretin lost potency on storage, as summarized in chapter 3. In most cases the Asp^3($\alpha\rightarrow\beta$)-rearrangement was stated to be responsible for this instability. In an attempt to obtain a "stable" secretin-like peptide we synthesized analogues of secretin in which Gly^4 had been replaced. We wish to describe here the synthesis of [Ala^4]-secretin and [Sar^4]-secretin, and their biological and immunological potency. In these peptides, Gly^4 in secretin, has been replaced by Ala (α-methylGly), or Sar (N-methylGly), respectively. Consequently, in these peptides a potential Asp^3($\alpha\rightarrow\beta$)-rearrangement will be slower than in secretin, because of the influence of the extra methyl group. [Ala^4]-secretin has also been synthesized and studied by other groups (33, 38, 39).

6.2 EXPERIMENTS AND RESULTS

Protected [Ala^4]-secretin was synthesized by the all-REMA method, as shown for protected secretin in Fig. 2.1b, in which Boc-Ala was coupled instead of Boc-Gly. Protected [Sar^4]-secretin was synthesized as shown in Fig. 6.1.

The synthesis of protected [Sar^4]-secretin by the all-REMA method gave, according to reverse-phase high-performance liquid chromatography, a considerable amount of a by-product (about 24%; UV detection at 260 nm), probably protected iBoc-[Sar^4]-S(4-27). Such problems have also been observed with the coupling of amino acid derivatives to proline-residues (80, and references cited herein). Here the problem was solved by coupling partially protected dipeptides with a C-terminal proline residue. This approach was not satisfactory in the synthesis of [Sar^4]-secretin, because of difficulties in the purification of the desired dipeptide Boc-Asp(OBzl)-Sar. Application of the approach shown in Fig. 6.1 resulted in a protected peptide having a quality comparable with that of protected secretin. The amino acid composition of both protected 27-peptides, after acidic hydrolysis, is given in Table 6.1.

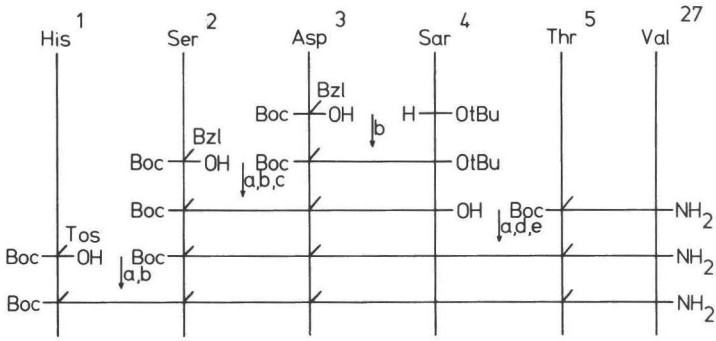

Fig. 6.1 The synthesis of protected [Sar^4]-secretin

a Deprotection by acidolysis in TFA.

b Coupling by the excess mixed anhydride method.

c Isolation of the product by extraction, followed by gel permeation chromatography.

d Coupling by the DCCI/HOBt method.

e Purification by by gel permeation chromatography.

Both protected peptides were deprotected by acidolysis with HF/anisole. The peptides were purified by ion-exchange chromatography, followed by preparative HPLC.

Experimental data are given in Table 6.2.

The end products were practically pure according to TLC (silica gel; butanol/pyridine/acetic acid/water 60:20:6:24) and HPLC (Polygosil C18; methanol/water/TFA 64:36:0.1). Chromatographic data of the peptides are presented in Table 6.3. The amino acid composition of these products, after acidic and enzymic hydrolysis, is given in Table 6.1.

Table 6.1 Amino acid analyses of analogues of secretin (S)

	[Ala^4]-S				[Sar^4]-S			
	a c	b c	b d	Theory	a c	b c	b d	Theory
Asp	1.79	2.02	2.00	2	1.63	2.07	1.04	2(1[g])
Thr	1.32	1.86		2	1.34	1.75		2
Ser	2.73	3.56		4	2.75	3.18		4
Sar[e]					0.58	1.16		1(0[g])
Glu	3.19	2.87	1.05	3(1[f])	2.79	3.14	0.98	3(1[f])
Gly	1.00	1.22	0.98	1	1.09	1.35	0.98	1
Ala	1.85	2.08	1.92	2	1.12	1.08	0.97	1
Val	1.13	0.98	0.92	1	1.02	1.05	1.02	1
Leu	6.00	5.82	6.05	6	6.17	5.48	6.10	6
Phe	0.76	0.95	1.03	1	0.81	0.95	0.86	1
His	0.79	1.04	1.07	1	0.82	0.98	0.99	1
Arg+Orn	3.81	4.01	3.98	4	3.82	3.90	3.91	4

a Crude protected peptide.

b Purified peptide.

c Acidic hydrolysis (6 M HCl, 24 h, 110 °C). Gln was determined as Glu.

d Enzymic hydrolysis (LAP, 24 h, 37 °C). Thr, Ser, and Gln could not be determined.

e Sar was eluted from the column (Kontron Liquimat III; Durrum resin DC 4A (25 x 0.4 cm I.D.); Pico buffer system II) between Ser and Glu. It was detected at 570 nm after reaction with ninhydrin, like the other amino acids.

f Glu = 1; Gln = 2.

g The Asp-Sar bond was not hydrolysed with LAP.

Table 6.2 Experimental data of the synthesis of analogues of secretin (S)

Peptide	Deprotection[a]		Purification			
	Starting material (mg)	Yield (mg)	Starting material (mg)[b]	Yield (mg)[b]	Starting material (mg)[c]	Yield (mg)[c,d]
[Ala^4]-S	88	70	67	17	4.5[e]	1.7
[Sar^4]-S	52	38	35	18	4.2[f]	0.6

a Treatment with HF/anisole; 1 h; 0 °C.

b Ion-exchange chromatography (SP-Sephadex C-25; 0.02 M NH_4HCO_3 (pH 6.1), followed by 0.05 M NH_4HCO_3 (pH 6.5)).

c HPLC; methanol/water/TFA 68:32:0.5; ambient temperature.

d Corrected for peptide content. All the other figures have not been corrected.

e LiChrosorb RP-18, 10 µm (20 x 0.8 cm I.D.).

f Polygosil C18, 10 µm (20 x 0.8 cm I.D.).

Table 6.3 Chromatographic data of secretin (S) and analogues

	S	[Ala^4]-S	[Sar^4]-S
R_f [a]	0.13	0.13	0.13
k' [b,c]	2.3	2.3	2.3
k' [b,d]	2.4	2.1	2.3

a TLC; silicagel; butanol/pyridine/acetic acid/water 60:20:6:24.

b HPLC; Polygosil C18, 10 µm (30 x 0.4 cm I.D.); temperature: 30 °C.

c Eluent: methanol/water/trifluoroacetic acid 64:36:0.1.

d Eluent: methanol/water 84:16 with 0.005 M perfluorooctanoic acid.

The analogues were assayed for their potency to stimulate the exocrine pancreatic secretion in the anaesthetized ferret and/or in the anaesthetized cat by Professor T. Scratcherd, Sheffield, England, and in the anaesthetized rat by Dr. T.E. Solomon, Los Angeles, CA, USA (Table 6.4).

Table 6.4 Biological potency of analogues of secretin (S)

Peptide	Biological potency[a] (%)	animal	n[b]	m[c]
[Ala^4]-S	10	cat[d]	3	1
	2	ferret[d]	2	2
	8	rat[e]	1	5
[Sar^4]-S	0.1	ferret[d]	1	1
	<0.4, >0.04	rat[e]	1	5

a The analogues were compared with pure secretin, the potency of which was assumed to be 100%.

b Number of assays per dose in one animal.

c Number of animals used.

d Only one dose was administered.

e Three doses were administered.

The immunological behaviour of the peptides is shown in Fig. 6.2. The radioimmunoassays were performed by Dr. T.M. Chang, Rochester, NY, USA.

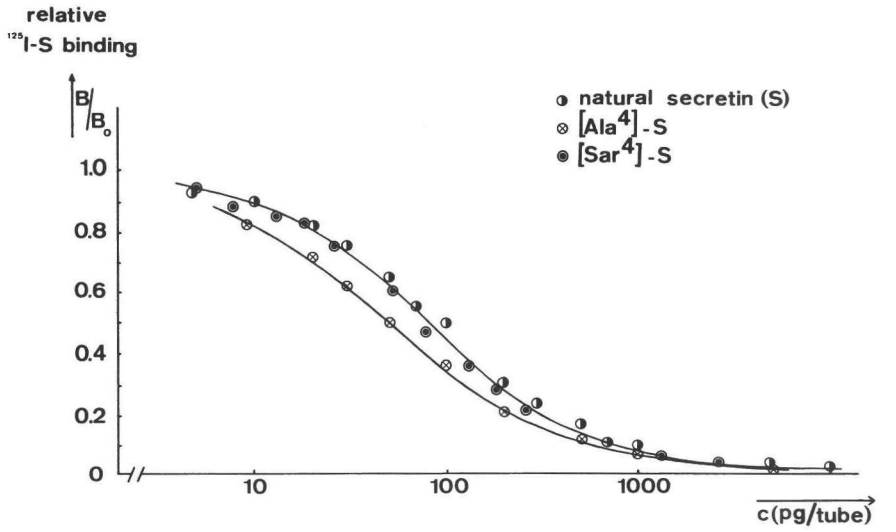

Fig. 6.2 The displacement of [^{125}I-His^{1}]-secretin from secretin antibody by natural secretin and synthetic secretin analogues (see chapter 2.2.7).

6.3 EXPERIMENTAL PART

Reagents, solvents, and amino acid derivatives were purchased, except for Sar-OtBu hydrochloride, which was synthesized following routine procedures (melting point = 136-137 °C, elemental analysis: calc.: C 46.28, H 8.88, N 7.71, Cl 19.52, found: C 46.4, H 8.9, N 7.7, Cl 19.4).

For the technique of coupling of protected amino acids by the REMA method, for the removal of protecting groups, and for the analysis and purification of protected and unprotected peptides we refer to chapter 2.

Bioassays were performed by Professor T. Scratcherd, Department of Physiology, University of Sheffield, Sheffield, England, with the anaesthetized ferret or the anaesthetized cat (58), and by Dr. T.E. Solomon, Center for Ulcer Research and Education (CURE), Veterans Administration, Los Angeles, CA, USA, with the anaesthetized rat. Radioimmunoassays were performed by Dr. T.M. Chang, The Genesee Hospital, The Isaac Gordon Center for Digestive Diseases and Nutrition, Rochester, NY, USA. Details are given in the chapters 2 and 5.

THE SYNTHESIS OF Boc-Asp(OBzl)-Sar-OtBu

Boc-Asp(OBzl) (3.2 g, 10 mmol) was dissolved in 10 ml of dimethylformamide (DMF). At -15 °C N-methyl morpholine (NMM, 1.11 ml, 10 mMol) and isobutyl chloroformate (1.20 ml, 9 mmol) were added. After 2 minutes, Sar-OtBu.HCl (0.88 g, 4.8 mmol) and NMM (0.53 ml, 4.8 mmol), dissolved in 20 ml of DMF/H_2O (1:1) were added. After stirring for 1 h, 20 ml of 2 M $KHCO_3$ was added and the reaction mixture was warmed to 0 °C. Then, 20 ml of water was added to the mixture, which was extracted with ethyl acetate. The organic mixture was washed with water, dried ($MgSO_4$) and evaporated after filtration. The resulting oil was dissolved in 10 ml of chloroform/methanol (6:1) and chromatographed on a column (20 x 2.5 cm I.D.) filled with aluminium oxide (Merck, Darmstadt, G.F.R.; activity I), with chloroform/methanol (6:1) as the eluent. The main fractions were collected and evaporated to dryness.
Yield: 1.74 g of oil (3.9 mmol; 81%).
Amino acid analysis: Asp 1.03, Sar 0.97.

THE SYNTHESIS OF Boc-Ser(Bzl)-Asp(OBzl)-Sar

Boc-Asp(OBzl)-Sar-OtBu (5.76 g, 12.8 mmol) was dissolved in 50 ml of TFA. After 60 minutes the solvent was evaporated, the resulting oil was triturated with ether and filtered, and the residue was dried.

Boc-Ser(Bzl) (4.3 g, 14.6 mmol) was dissolved in 25 ml of DMF. At -15 °C NMM (1.6 ml, 14.4 mmol) and isobutyl chloroformate (1.8 ml, 13.5 mmol) were added. After 3 minutes, Asp(OBzl)-Sar, obtained as described above and dissolved in 25 ml of DMF, was added. The pH was adjusted to pH ~ 8 with NMM. After 45 minutes 10 ml of 2 M $KHCO_3$ was added and the reaction mixture was stirred at 0 °C for 30 minutes. Then water was added, the pH was adjusted to pH ~ 3 with citric acid, and the mixture was extracted with ethyl acetate. The organic layer was washed with water, dried ($MgSO_4$), and evaporated to dryness after filtration.
Yield: 7.05 g of oil.

A part of this oil (0.52 g) was dissolved in DMF and purified by gel permeation chromatography (Sephadex LH-20 (90 x 2.5 cm I.D.); DMF). The main fractions were collected and evaporated to dryness.
Yield: 0.32 g of oil (0.6 mmol; overall yield: 58%).

The product appeared to be rather pure, according to TLC and reverse-phase HPLC. Amino acid analysis: Asp 0.99, Ser 0.75, Sar 1.01.

THE SYNTHESIS OF PROTECTED [SAR^4]-S(2-27)

Boc-Ser(Bzl)-Asp(OBzl)-Sar (0.17 g, 0.30 mmol) was dissolved in 2.5 ml of DMF. Dicyclohexylcarbodiimide (52 mg, 0.25 mmol) and N-hydroxybenzotriazole.H_2O (45 mg, 0.30 mmol) were added at 0 °C. After stirring for 1 hour at ambient temperature, side-chain protected S(5-27), obtained in the all-REMA synthesis of secretin (chapter 2; 172 mg, 0.05 mmol), dissolved in 2.5 ml of DMF, was added. The solution was neutralized with NMM, after which 6 µl of NMM (0.05 mmol) was added. After stirring for 21 h at ambient temperature, the mixture was clarified by centrifugation and the solution was chromatographed (Sephadex LH-20 (90 x 2.5 cm I.D.); DMF). The main product was concentrated and precipitated by the addition of saline. After filtration, the peptide was dried in vacuo.
Yield: 156 mg (0.04 mmol, 82%). The quality of this product was comparable with that of protected S(2-27), obtained by the all-REMA method, according to reverse phase HPLC. Amino acid analysis: Asp 1.58, Thr 1.39, Ser 2.89, Sar 0.81, Glu 2.95, Gly 1.03, Ala 1.10, Val 1.03, Leu 6.21, Phe 0.79, Arg+Orn 3.80.

6.4 CONCLUSIONS

Two analogues of porcine secretin, [Ala^4]-secretin and [Sar^4]-secretin, were synthesized. Both peptides possess a considerably lower potency to stimulate the exocrine pancreatic activity than secretin in the animals used. [Ala^4]-secretin still possessed a rather high potency. [Sar^4]-secretin was much less potent than secretin in both the ferret and the rat. These peptides and secretin had similar immunological properties in the system used.

These results show that [Ala^4]-secretin might be used as a "stable" secretin-like material. [Sar^4]-secretin is less suited for this purpose because of its much lower potency. The necessity of having a "stable" secretin-like material, however, is questionable, in view of the results of our research on the instability of secretin, as described in chapter 3, and those of Jaeger et al. (16, 64). Because of this we did not study the stability of the analogues described here.

7. SYNTHESIS AND SOME PROPERTIES OF ANALOGUES OF SECRETIN AND THE VASOACTIVE INTESTINAL PEPTIDE (VIP): THE VASECTRINS

7.1 INTRODUCTION

The discovery of a series of peptides isolated from the gastrointestinal tract mostly of hogs, uncovered great sequence similarities in some peptides. This favored the classification into peptide families. Gastrin, cholecystokinin, and caerulein (isolated from amphibian skin) form the gastrin family, while secretin, glucagon, the vasoactive intestinal peptide (VIP), and the gastric inhibitory peptide (GIP) are the members of the secretin family (Fig. 7.1) (21, 24, 25). Recently another 27-peptide, called PHI, was added to this family (see chapter 1.3).

Secretin and VIP, especially, not only share several sequence similarities but have also several closely related biological effects. Much work has already been done to localize those parts of the molecules that are responsible for distinct properties (81-85).

We wish to describe here the synthesis and some properties of analogues of secretin modified in such a way that their sequences are intermediate between those of porcine secretin and porcine VIP. We call these analogues vasectrins (VS).

We synthesized [Ala^4]-secretin (VS I), [Ala^4,Val^5]-secretin (VS II), and [Ala^4,Val^5,Asp^8,Asn^9,Tyr^{10},Thr^{11}]-secretin (VS III) (Fig. 2) for several reasons. Firstly, porcine VIP is thought to be a more stable peptide than secretin, although recently an Asn^{28} deamidation during storage as a lyophilized product has been postulated (86). Experiments with the vasectrins might help to elucidate the mechanism of the inactivation of secretin. Secondly, these peptides would allow a study of structure-activity relationships in the secretin family. Finally, problems in the synthesis of porcine VIP could be studied during the synthesis of the vasectrins.

The peptides [Ala^4]-secretin and [Ala^4,Val^5]-secretin have also been prepared and studied by others (16, 33, 38, 39, 84).

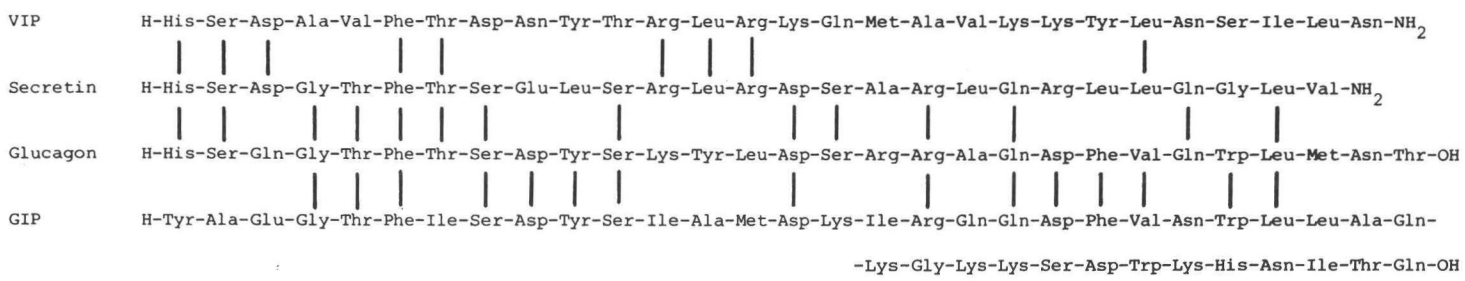

Fig. 7.1 The primary structure of VIP, secretin, glucagon, and GIP, isolated from hogs. The vertical lines indicate that in those positions identical amino acid residues are present in neighboring peptides.

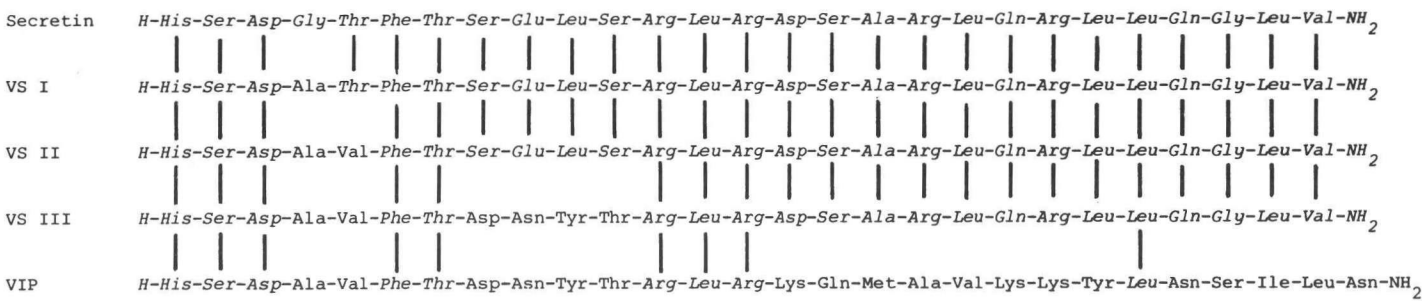

Fig. 7.2 The primary structure of porcine secretin, the vasectrins, and porcine VIP. The vertical lines indicate that in those positions identical amino acid residues are present in secretin.

7.2 RESULTS AND DISCUSSION

Fully protected VS I and VS II were synthesized by the all-Repetitive Excess Mixed Anhydride (REMA) method as described for secretin (chapter 2). These protected peptides were analysed by reverse-phase high-performance liquid chromatography and amino acid analysis after acidic hydrolysis (Table 7.1). The quality of the products was comparable with that of synthetic secretin. Partially protected VS III was synthesized by coupling of the segment VIP(1-13) to secretin(14-27)-NH_2, followed by purification by gel permeation chromatography. The product obtained had a satisfactory amino acid composition after acidic hydrolysis (Table 7.1)

The protected peptides VS I and VS II were deprotected by acidolysis with hydrogen fluoride (HF)/anisole. Protected VS III was deprotected by subsequent acidolysis with trifluoroacetic acid (TFA) and HF/anisole.

VS I and VS II were purified by ion-exchange chromatography, followed by preparative HPLC. VS III was purified by preparative HPLC.

The products obtained were practically pure according to TLC (silica gel; butanol/pyridine/acetic acid/water 60:20:6:24) and HPLC (Polygosil C18; methanol/water/TFA 63:37:0.1). Chromatographic data of the peptides are presented in Table 7.2. The amino acid compositions of the vasectrins, after acidic and enzymic hydrolysis, were consistent with calculated values (Table 7.1). Amino acid analysis of VS III, after acidic hydrolysis followed by treatment with L-amino acid oxidase (LAO), showed that no racemization had occurred during the segment coupling.

Table 7.2 Chromatographic data of secretin and the vasectrins

	Secretin	VS I	VS II	VS III
R_f [a]	0.13	0.13	0.15	0.13
k' [b,c]	4.6	4.4	5.7	2.9
k' [b,d]	2.4	2.1	2.2	2.4

a TLC; silicagel; butanol/pyridine/acetic acid/water 60:20:6:24.
b HPLC; Polygosil C18, 10 µm (30 x 0.4 cm I.D.); temperature: 30 °C.
c Eluent: methanol/water/TFA 63:37:0.1.
d Eluent: methanol/water 84:16 with 0.005 M perfluorooctanoic acid.

The syntheses of VS I and VS II are further proofs, along with the synthesis of secretin, that 27-peptides, even with the sterically bulky sequence

Table 7.1 Amino acid analyses of the vasectrins

	VS I				VS II				VS III			
	a c	b c	b d	Theory	a c	b c	b d	Theory	a c	b c	b d	Theory
Asp	1.79	2.02	2.00	2	1.82	2.05	1.98	2	4.38	3.99	2.85	4(3^f)
Thr	1.32	1.86		2	0.89	0.99		1	2.30	1.86		2
Ser	2.73	3.56		4	3.16	2.77		4	2.08	1.84		2
Glu	3.19	2.87	1.05	3(1^e)	3.07	3.11	1.10	3(1^e)	1.87	2.10		2(0^g)
Gly	1.00	1.22	0.98	1	1.04	1.07	1.04	1	1.00	1.15	1.08	1
Ala	1.85	2.08	1.92	2	1.94	2.06	2.07	2	2.11	2.10	2.01	2
Val	1.13	0.98	0.92	1	1.88	1.97	1.90	2	2.13	1.95	1.91	2
Leu	6.00	5.82	6.05	6	6.11	5.87	5.94	6	4.93	4.86	5.21	5
Tyr									0.66	0.90		1
Phe	0.76	0.95	1.03	1	0.91	0.95	1.03	1	1.14	0.96	0.94	1
His	0.79	1.04	1.07	1	0.78	1.00	0.97	1	1.18	0.94	0.94	1
Arg+Orn	3.81	4.01	3.98	4	3.77	3.93	3.97	4	3.95	3.95	4.05	4

a Crude protected product.

b Purified unprotected peptides.

c Acidic hydrolysis (6 M HCl, 24 h, 110 °C). Gln was determined as Glu.

d Enzymic hydrolysis (LAP, 24 h, 37 °C). Asn, Gln, Thr, Ser, and Tyr cannot be determined.

e Glu = 1, Gln = 2.

f Asp = 3, Asn = 1.

g Glu = 0, Gln = 2.

-Ala-Val-Phe-, like in VS II, can easily be synthesized by the all-REMA method. The results with the synthesis of VS III indicate that our strategy for the synthesis of porcine VIP, coupling of the two segments, VIP(1-13) and VIP(14-28)-NH_2, is a promising one. The main problem is the synthesis of VIP(14-28)-NH_2 (88), because of extreme low solubility of intermediate peptides, as already reported by Klausner and Bodanszky (89) and Wendlberger et al. (90).

The vasectrins were assayed for their potency to stimulate the exocrine pancreatic secretion in the anaesthetized cat, and/or in the anaesthetized ferret, by Professor T. Scratcherd, Sheffield, England, and in the anaesthetized rat, by Dr. T.E. Solomon, Los Angeles, CA, USA (Table 7.3). All three vasectrins were less potent than secretin itself but each analogue was clearly able to stimulate pancreatic secretion. VS I and VS II appeared to be full agonists for stimulation of pancreatic secretion in the rat since they produced nearly maximal flow rates when given at high doses. The situation for VS III was less clear, since it did not appear to produce maximal flow rates in the rat. This is similar to the effect of VIP in the rat (91), but extensive further testing would be required to confirm this conclusion.

Table 7.3 The biological potency of the vasectrins

Peptide	Biological potency (%)[a]	animal	n[b]	m[c]
VS I	10	cat[d]	3	1
	2	ferret[d]	2	2
	8	rat[e]	1	5
VS II	1	ferret[d]	2	2
	31	rat[e]	1	6
VS III	1	cat[d]	5	1
	active	rat[e]	1	5

a The analogues were compared with pure secretin, the potency of whichwas assumed to be 100%.

b Number of assays per dose in one animal.

c Number of animals used.

d Only one dose was administered.

e See text; three doses were administered.

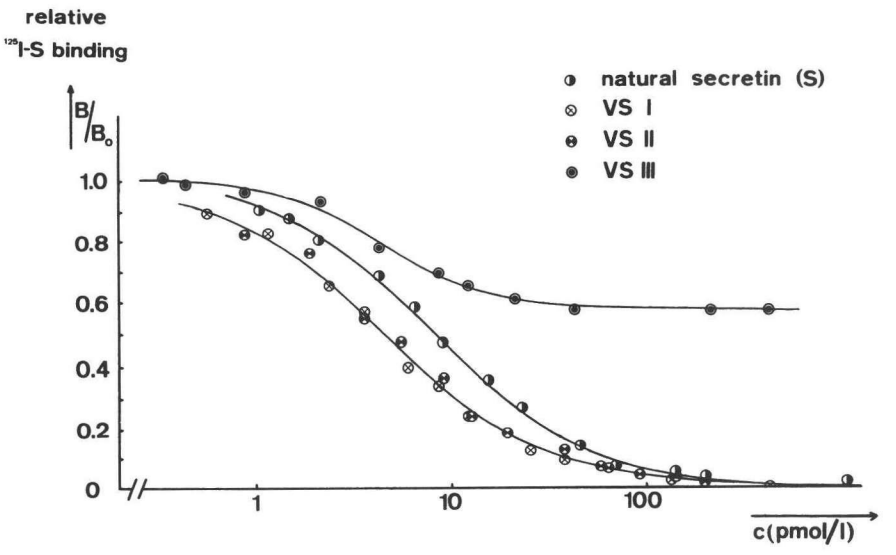

Fig. 7.3 The displacement of [^{125}I-His^{1}]-secretin from secretin antibody by natural secretin and the vasectrins.

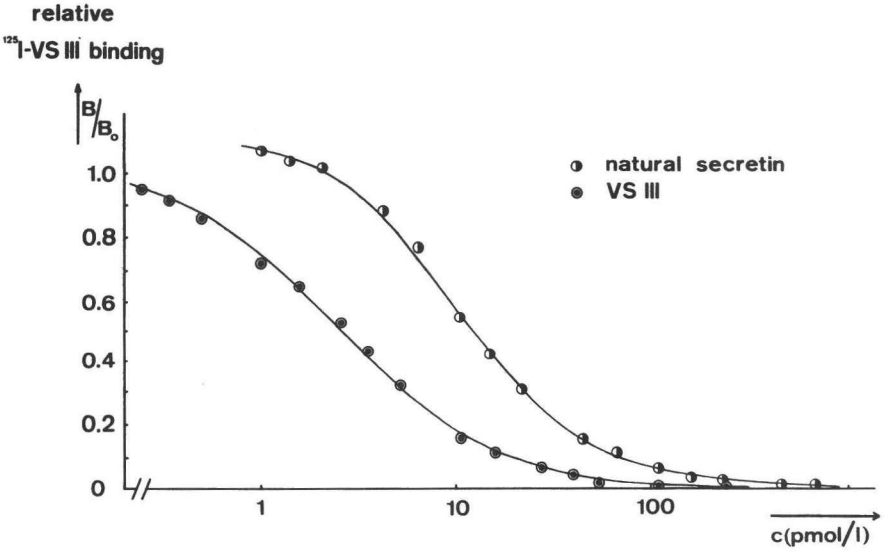

Fig. 7.4 The displacement of [^{125}I-His^{1}]-VS III from secretin antibody.

Table 7.4 Experimental data of the synthesis of the vasectrins

Peptide	Deprotection[a]		Purification			
	Starting material (mg)	Yield (mg)	Starting material (mg)[b]	Yield (mg)[b]	Starting material (mg)[c]	Yield (mg)[c,d]
VS I	88	70	67	17	4.5[e]	1.7
VS II	251	225	97	34	5.0[e]	1.9
VS III	25	18			6.7[f]	0.7

a Treatment with HF/anisole; 1 h; 0 °C.

b Ion-exchange chromatography (SP-Sephadex C-25; 0.02 M NH_4HCO_3, pH 6.1, followed by 0.05 M NH_4HCO_3, pH 6.5).

c HPLC; ambient temperature.

d Corrected for peptide content. All the other figures have not been corrected.

e LiChrosorb RP-18, 10 µm (20 x 0.8 cm); methanol/water/TFA 68:32:0.5.

f Nucleosil C18, 5 µm (15 x 0.4 cm); methanol/water/TFA 60:40:0.5.

The immunological behaviour of the vasectrins was studied by Dr. T.M. Chang, Rochester, NY, USA. Fig. 7.3 shows the displacement of secretin from secretin antibody by the vasectrins. Fig. 7.4. shows the displacement of VS III from secretin antibody by secretin. It was found that VS III also shows strong interactions with VIP antibody.

Secretin, VS I, and VS II therefore all show rather similar immunoreactivity to the secretin antibody used. The modifications in the N-terminal sequence 1-5 in secretin do not significantly affect the major secretin antigenic determinant(s). VS III, however, exhibits a different immunological behaviour. The figures obtained indicate that alterations in the sequence 8-11 in secretin, or a part of this, cause the loss of an important antigenic determinant. Still, VS III shows strong interactions with approximately 50% of the binding sites of the secretin antibody. The half-displacement dose of VS III was similar to that of secretin (Fig. 7.3). Consequently, the VS III binding to about 50% of the ^{125}I-secretin binding sites involve antibody binding sites totally competitive with secretin, with an affinity similar to overall secretin binding to the antibody. The cross-reactivity of natural VIP toward the secretin antibody used was less than 0.5%. Consequently, the C-terminal sequence of secretin contains (at least) one important antigenic determinant. These results support the "two antigenic domain model", as discussed recently by Chang and Chey (57).

Since VS III also shows strong interaction with VIP antibody, this peptide contains (at least) one antigenic determinant of VIP. Since secretin does not interact with the VIP antibody used, VIP has an important antigenic determinant in its sequence 1-13.

The stability of the vasectrins was hardly studied, because of our find that in secretin some intramolecular rearrangements, e.g. the Asp^3($\alpha\rightarrow\beta$)-rearrangement, often thought to be responsible for the instability, only proceed very slowly (see chapter 3).

7.3 EXPERIMENTAL PART

Reagents, solvents, and amino acid derivatives were purchased.

For the technique of coupling of protected amino acids by the REMA method, for the removal of protecting groups, and for the analysis and purification of protected and unprotected peptides we refer to chapter 2.

Partially protected VS III was synthesized by coupling of the segments Boc-His-Ser(tBu)-Asp-(OtBu)-Ala-Val-Phe-Thr-Asp(OBzl)-Asn-Tyr-Thr(Bzl)-Arg(NO_2)-Leu (I, partially protected VIP(1-13)) to Boc-Arg(NO_2)-Asp(OBzl)-

Ser(Bzl)-Ala-Arg(NO_2)-Leu-Gln-Arg(NO_2)-Leu-Leu-Gln-Gly-Leu-Val-NH_2 (II, protected secretin (14-27)-NH_2), after treatment with TFA. The synthesis of I has been described by Buijen van Weelderen (88). II was obtained during the synthesis of secretin (chapter 2).

II (120 mg; 60 µmol) was dissolved in 5 ml of TFA. After 20 min, the solution was concentrated in vacuo and triturated with ether. After filtration, the material was dissolved in 5 ml of DMF and precipitated by the addition of 25 ml of 0.5 M $KHCO_3$. The precipitate was filtered off, washed with water, and dried.

Yield: 87 mg of 14-peptide (43 µmol; 72%).

I (75 mg; 37 µmol) was dissolved in 1.5 ml of DMF. At 0 °C, HOBt (6.1 mg; 45 µmol) and DCCI (7.4 mg; 36 µmol) were added. After stirring for 1 h at 0 °C and 1 h at 20 °C the 14-peptide described above (50 mg; 25 µmol) was added. After 48 h, II (38 mg; 19 µmol), activated as described above, was added to the reaction mixture. After another 120 h the mixture was chromatographed in two portions (Merckogel OR PVA 20000 (100 x 2.5 cm); DMF). The main fractions were collected, evaporated in vacuo and lyophilized from acetic acid.

Yield: 25 mg (6 µmol; 25%).

Amino acid analysis: see Table 7.1.

The material obtained was dissolved in 5 ml of TFA. After 5 h, acetic acid was added and the material was lyophilized. This material was used for HF/anisole treatment.

The enzymic hydrolysis with L-amino acid oxidase (LAO) was performed as follows: To the hydrolyzed peptide (6 N HCl, 24 h, 110 °C), dissolved in 0.2 M "TRIS-buffer" (pH 7.5) (500 mmol/ml), a solution of L-amino acid oxidase (Crotalus Adamanteus, Sigma, St. Louis, MO, USA) 0.25 units/µmol digestable amino acid) and 5 µl of toluene were added, and the solution left at 37 °C for 24 h in an oxygen atmosphere. The resulting mixture was analysed with the amino acid analyser.

Bioassays were performed by Professor T. Scratcherd, Department of Physiology, University of Sheffield, Sheffield, England, with the anaesthetized cat (57) or the anaesthetized ferret, and by Dr. T.E. Solomon, Center for Ulcer Research and Education (CURE), Veterans Administration, Los Angeles, CA, USA, with the anaesthetized rat. Radioimmunoassays were performed by Dr. T.M. Chang, The Genesee Hospital, The Isaac Gordon Center for Digestive Diseases and Nutrition, Rochester, NY, USA. Details are given in the chapters 2 and 5.

SUMMARY

Secretin is a hormone found in the mucosa of the upper part of the intestine of many animals and of man. The principal stimulus releasing the hormone into the blood is the acidification of the contents of the upper intestine. The most important biological effect is the stimulation of the pancreas to secrete a basic, enzymes-containing juice.

Secretin appeared to be applicable for curing ulcers in the gastro-intestinal tract and as a diagnosticum, e.g. for the pancreatic function. A considerable problem in this context, however, was the instability of the hormone in vitro. A number of researchers mentioned the fact that the biological potency of secretin decreased rapidly, especially in an aqueous solution. The object of the investigations described in this thesis was to ascertain the cause(s) of the inactivation of porcine secretin in vitro at the molecular level and the finding of a practical remedy.

In chapter 1 a general review of secretin is presented. Secretin was the first hormone to be recognized as such, in 1902, by Bayliss and Starling. It was not until 1961 that the hormone was isolated from porcine intestine. It was found to be a linear 27-peptide amide (Fig. 1):

His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-
1 2 3 4 5 6 7 8 9 10 11 12 13 14
-Asp-Ser-Ala-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH_2
15 16 17 18 19 20 21 22 23 24 25 26 27

Fig. 1 The sequence of porcine secretin.

The peptide was synthesized several times, inter alia in this laboratory. The secondary and tertiary structure were studied using ORD-,CD-,and NMR-spectroscopy and electron microscopy.The peptide is found to be present in solution in a preferred conformation, in which it partly occurs as an α-helix. Several analogues,partial sequences,and analogues of the latter have been synthesized.

In chapter 2 the synthesis of secretin via the repetitive excess mixed anhydride (REMA) method is discussed. In order to make research on secretin possible it is important that a sufficiently large quantity of secretin should be available. This can only be obtained by means of synthesis. In this laboratory Van Zon and Beyerman developed a good synthesis with the aid of the REMA method. This synthesis route was later modified by us on some points. A number of protective groups were altered, while the ultimate deprotection of the protected peptide was effected by acidolysis in liquid hydrogen fluoride/anisole. The synthesis was monitored completely by reverse-phase high-performance liquid chromatography, for which a number of systems were developed.

In chapter 3 the investigation on the loss of biological potency of secretin in vitro is described. A good many researchers reported that secretin was not stable. Especially in an aqueous solution the biological potency of secretin was sometimes rapidly lost. From the different data, however, no clear picture could be gained of the circumstances under which the potency was lost. Experiments with secretin synthesized by us, in co-operation with Professor T. Scratcherd, Sheffield, England, and Professor M.I. Grossman, Los Angeles, CA, USA, also produced contradictory results. Ultimately the secretin potency in an aqueous solution was found to decrease hardly, if at all, when measures were taken to prevent adsorption on the wall of the vessel in which the solution was stored, by means of certain additions. Secretin was found to have a strong tendency to adsorb on different materials.

A number of reactions that may occur in peptides are known. One of these reactions, the $Asp^{3}(\alpha\rightarrow\beta)$-Gly-rearrangement (Fig. 2), was generally considered to be responsible for the instability of secretin. Some reactions were studied, at first in model peptides, later in secretin. The $Asp^{3}(\alpha\rightarrow\beta)$-Gly-rearrangement was found to proceed slowly in secretin. In a weakly acidic medium at the same time a splitting of the Asp^{3}-Gly peptide chain was found to occur. Indications were also found, after research on model peptides and secretin, that an $Asp^{15}(\alpha\rightarrow\beta)$-Ser-rearrangement occurred. A number of other conceivable reactions could not be shown to occur in secretin.

The rapid loss of biological potency in a solution of secretin therefore is probably due in particular to a reversible adsorption on glass or synthetic materials. This may be prevented by the use of certain additions. A number of chemical reactions proceed only slowly. Some of these reactions cause a loss of biological potency, as is shown in chapter 5.

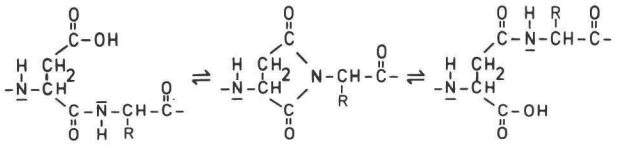

Fig. 2 The Asp(α→β)-rearrangement in secretin.
Asp^3: R=H; Asp^{15}: R=CH_2OH

In chapter 4 the synthesis and some properties are described of S(1-6)-NH_2, [β-Asp^3]-S(1-6)-NH_2, [aspartoyl^3]-S(1-6)-NH_2, S(4-6)-NH_2, S(13-18), [β-Asp^{15}]-S(13-18), S(13-14), and S(15-18). These peptides were used in the investigation into the behaviour of partial sequences of secretin in an aqueous solution, as described in chapter 3.

In chapter 5 the synthesis, the biological and immunological behaviour, and some chromatographic properties are described of [β-Asp^3]-secretin, [aspartoyl^3]-secretin, and secretin(4-27). These peptides were used in the investigation into the behaviour of secretin in an aqueous solution, as described in chapter 3.

In chapter 6 the synthesis and the biological and immunological behaviour are described of [Ala^4]-secretin and [Sar^4]-secretin. In these peptides the Asp^3(α→β)-Gly-rearrangement will proceed less rapidly than in secretin, so that, if desired, they might be used as "stable" secretin preparations. Both peptides, however, were found to be much less potent than secretin.

In chapter 7 the synthesis and the biological and immunological behaviour are described of three analogues of secretin which have been modified in such a way that their sequences are more similar to that of the vasoactive intestinal peptide (VIP). VIP is a gastrointestinal peptide greatly resembling secretin as far as the primary structure and the physiological/pharmacological behaviour are concerned. The synthesized peptides, called vasectrins by us, were synthesized (1) to obtain "stable" secretin preparations and (2) to study problems in the synthesis of VIP. With the analogues various experiments were carried out, as a result of which a more profound insight into the immunological properties of secretin was gained.

The research on secretin, partly described in this thesis, has been published or is in the press (28, 93-103).

SAMENVATTING

Secretine is een hormoon dat voorkomt in de mucosa van het bovenste gedeelte van de darm in vele dieren en de mens. De voornaamste prikkel die het hormoon doet vrijkomen in het bloed is het zuur worden van de bovenste darminhoud. De belangrijkste biologische werking is het stimuleren van de pancreas tot het uitscheiden van een basische, enzymen bevattende vloeistof.

Secretine leek toepasbaar als geneesmiddel bij zweren van het maag-darm-kanaal en als diagnosticum, bijvoorbeeld voor de pancreasfunctie. Een groot probleem hierbij was evenwel de instabiliteit van het hormoon in vitro. Een aantal onderzoekers maakte melding van het feit dat de biologische activiteit van secretine snel afnam, vooral in waterige oplossing. Het doel van het in dit proefschrift beschreven onderzoek was het vinden van de oorzaak of de oorzaken van de desactivering van secretine in vitro op het moleculaire niveau en het vinden van een practische oplossing.

Hoofdstuk 1 geeft een algemeen overzicht van secretine. Secretine was het eerste hormoon dat als zodanig werd herkend, in 1902, door Bayliss en Starling. Pas in 1961 werd het hormoon geisoleerd uit varkensdarm. Het bleek een lineair 27-peptide amide te zijn (Fig. 1).

His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-
1 2 3 4 5 6 7 8 9 10 11 12 13 14
-Asp-Ser-Ala-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH_2
15 16 17 18 19 20 21 22 23 24 25 26 27

Fig. 1 De sequentie van varkenssecretine.

Het peptide werd meerdere malen gesynthetiseerd, onder andere in Delft. De secundaire en tertiaire structuur werden bestudeerd met ORD-, CD- en NMR-spectroscopie en electronen microscopie. Het peptide blijkt in oplossing voor te komen in een voorkeursconformatie, gedeeltelijk als α-helix. Een groot aantal analoga, deelsequenties en analoga hiervan, zijn gesynthetiseerd.

In hoofdstuk 2 wordt de synthese van secretine met behulp van de repetitieve overmaat gemengd anhydride methode besproken. Teneinde onderzoek aan secretine te kunnen doen is het van belang over een voldoend grote hoeveelheid ervan te beschikken. Deze kan alleen worden verkregen door middel van synthese. Op dit laboratorium werd door Van Zon en Beyerman een goede synthese ontwikkeld met behulp van de repetitieve overmaat gemengd anhydride methode. Deze synthese werd door ons op enkele punten gewijzigd. Een aantal beschermende groepen werd veranderd terwijl de uiteindelijke ontscherming van het beschermde peptide - voorheen katalytische hydrogenolyse - thans plaatsvond door acidolyse in vloeibare waterstoffluoride/anisool. De synthese werd geheel vervolgd met reverse-phase hoge druk vloeistofchromatografie, waarvoor een aantal systemen werd ontwikkeld.

In hoofdstuk 3 wordt het onderzoek naar het verlies aan biologische activiteit van secretine in vitro beschreven. Een aanzienlijk aantal onderzoekers meldde dat secretine niet stabiel was. Vooral in waterig milieu ging de biologische activiteit van secretine soms snel verloren. Uit de verschillende gegevens was echter geen duidelijk beeld te vormen onder welke omstandigheden de activiteit verloren ging.

Experimenten met door ons gesynthetiseerd secretine, in samenwerking met Professor T. Scratcherd, Sheffield, Engeland en Professor M.I. Grossman, Los Angeles, CA, USA, gaven ook tegenstrijdige resultaten. Uiteindelijk bleek de secretineactiviteit in waterige oplossing niet of nauwelijks af te nemen als maatregelen genomen werden om adsorptie van het peptide, aan de wand van het vaatje waarin de oplossing werd bewaard, tegen te gaan door bepaalde toevoegingen. Secretine bleek een sterke neiging te bezitten aan verschillende materialen te adsorberen.

Een aantal reacties is bekend die kunnen optreden in peptiden. Een van deze reacties, de $Asp^3(\alpha\rightarrow\beta)$-Gly-omlegging (Fig. 2), werd algemeen verantwoordelijk geacht voor de instabiliteit van secretine. Enkele reacties werden bestudeerd, aanvankelijk in modelpeptiden, later in secretine. De $Asp^3(\alpha\rightarrow\beta)$-Gly-omlegging bleek langzaam op te treden in secretine. In zwak zuur milieu bleek tevens een Asp^3-Gly peptideketensplitsing op te treden. Ook werden aanwijzingen gevonden, na onderzoek aan modelpeptiden en secretine, dat een $Asp^{15}(\alpha\rightarrow\beta)$-Ser-omlegging optrad. Een aantal andere denkbare reacties kon niet worden aangetoond in secretine.

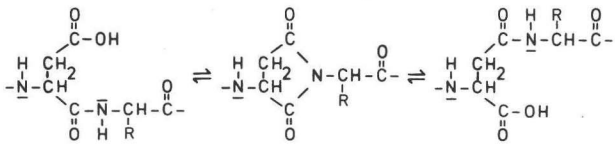

Fig. 2 De Asp(α→β)-omlegging in secretine.
Asp^3: R=H; Asp^{15}: R=CH_2OH

Het snelle verlies aan biologische activiteit in een oplossing van secretine wordt dus waarschijnlijk vooral veroorzaakt door een reversibele adsorptie aan glas of kunststoffen. Dit kan worden voorkomen door bepaalde toevoegingen te doen. Enkele chemische reacties treden slechts langzaam op. Sommige van deze reacties veroorzaken een verlies aan biologische activiteit zoals in hoofdstuk 5 blijkt.

In hoofdstuk 4 worden de synthese en enkele eigenschappen beschreven van S(1-6)-NH_2, [β-Asp^3]-S(1-6)-NH_2, [aspartoyl^3]-S(1-6)-NH_2, S(4-6)-NH_2, S(13-18), [β-Asp^{15}]-S(13-18), S(13-14) en S(15-18). Deze peptiden werden gebruikt bij het onderzoek naar het gedrag van deelsequenties van secretine in waterige oplossing, zoals beschreven in hoofdstuk 3.

In hoofdstuk 5 worden de synthese, de biologische en immunologische activiteit en enkele chromatografische eigenschappen beschreven van [β-Asp^3]-secretine, [aspartoyl^3]-secretine en secretine(4-27). Deze peptiden werden gebruikt bij het onderzoek naar het gedrag van secretine in waterige oplossing, zoals beschreven in hoofdstuk 3.

In hoofdstuk 6 worden de synthese en de biologische en immunologische activiteit beschreven van [Ala^4]-secretine en [Sar^4]-secretine. In deze peptiden zal de Asp^3(α→β)-Gly-omlegging minder snel optreden dan in secretine, zodat ze eventueel als "stabiel" secretinepreparaat zouden kunnen worden gebruikt. Beide peptiden bleken evenwel veel minder actief te zijn dan secretine.

In hoofdstuk 7 worden de synthese en de biologische en immunologische activiteit beschreven van drie analoga van secretine die zodanig zijn gemodificeerd dat hun sequenties meer op die van het vasoactive intestinal peptide (VIP) lijken. VIP is een gastrointestinaal peptide dat veel op secretine lijkt, qua primaire structuur en qua fysiologisch/farmacologisch gedrag.

De gesynthetiseerde peptiden, door ons vasectrines genoemd, werden gesynthetiseerd om "stabiele" secretinepreparaten te verkrijgen en problemen bij de synthese van VIP te bestuderen. Met de analoga werden diverse experimenten uitgevoerd waardoor een dieper inzicht in vooral de immunologische eigenschappen van secretine werd verkregen.

Het onderzoek aan secretine, gedeeltelijk beschreven in dit proefschrift, is gepubliceerd of in druk (28, 93-103).

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