115. Synthesis of the Gastrointestinal Peptide Hormone Secretin by the Repetitive Excess Mixed Anhydride (REMA) Method

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Summary. The REMA method was found to be very suitable for the synthesis of secretin. The procedure was rapid, since on a 0.2 mmol-scale the rate of one amino acid per day could be attained, and yielded an unambiguous product after simple ion-exchange chromatography. REMA-secretin was found to be chemically identical with secretin prepared by fragment condensations and showed a biological activity of 3.4 (2.9–4.3) clinical units/μg, comparable to that of the natural product (4 clinical units/μg). The yield of purified secretin, a heptacosapeptide amide, calculated on the basis of C-terminal residue, amounted to 5%.

1. Introduction. – By the method which one of us has called the repetitive excess mixed anhydride or REMA method [1] a nonapeptide was synthesized by *Tilak* [2]. Subsequently we effected by this method the complete synthesis of sequence 1–10 of human growth hormone (HGH 1–10) [3], the luteinising hormone-releasing hormone (LH-RH, or Gn-RH) [4], also a decapeptide, substance P [5], an undecapeptide, and sequences of human calcitonin of approximately the same size [6].

In order to ascertain the maximum limit for chain length of pure REMA-synthesized peptides, the synthesis of secretin (*Scheme 1*), a gastrointestinal hormone composed of 27 amino acid residues, was now undertaken[2])[3]). In a previous communication we reported on the REMA-synthesis of the C-terminal protected hexadecapeptide of secretin [10]. The limit for chain length of a purifiable peptide, obtained by sequential synthesis using the solid phase method [11] [12], had already been reached.

Scheme 1. (*cf.* [8].) *The amino acid sequence of the* (*porcine*) *hormone secretin*

H-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

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[1]) Taken, in part, from the Ph. D. Thesis of *A. van Zon*, Technische Hogeschool, Delft, 1974.

[2]) Standard abbreviations are used for amino acids and protecting groups [7]. In addition, CHA for cyclohexylalanine, DCHA for dicyclohexylamine, DMF for N,N-dimethylformamide, DMSO for dimethyl sulfoxide, NMM for N-methylmorpholine, TFA for trifluoroacetic acid, and TLC. for thin-layer chromatography.

[3]) Communicated in part in a lecture (*H.C.B.*) Basler Chemische Gesellschaft, February 21st, 1974; published in abbreviated form [8], and in a preliminary communication [9].

2. Fundamental precepts for sequential synthesis. – Stepwise elongation couplings should take place as completely as possible, since otherwise failure sequences [13] arise. A side-reaction occurring in the REMA-method in some cases, *e.g.* in the reaction with proline, involves coupling on the undesirable side of the mixed anhydride [3] [14]. If this so-called anomalous coupling occurs, it gives rise to an N-terminally, permanently-blocked peptide, a so-called truncated sequence [13]. As the synthesis continues the molecular weight of any truncated peptide will differ more and more from that of the peptide desired so that purification seems preferable only at a later stage. The difference in basicity of the various species on elimination of protecting groups and, in consequence, the purification by ion-exchange chromatography, will be discussed below. However, anomalous coupling to a considerable extent in a particular step, as established after deblocking, has to be avoided, just as in the case of coupling to prolyl peptides [3].

In order to ascertain the necessary stepwise yields in a sequential (seq.) synthesis such as that the number of failure sequences does not preclude a reasonable yield of secretin, the statistical distribution of the peptide mixture formed during its stepwise synthesis was determined as a function of the constant coupling yield P, according to *Baas et al.* [15]. The results are shown in Fig. 1. From this it appears, for instance, that with a constant coupling yield of 99.9% per step, the total mol-percentage of secretin will be 97.4, while with a constant coupling yield of 99.8% it is only 94.9%. The by-products then consist of various failure sequences with chain length of 26, 25 *etc.* amino acid residues. By use of fluorescamine [16] the disappearance of 99.9–

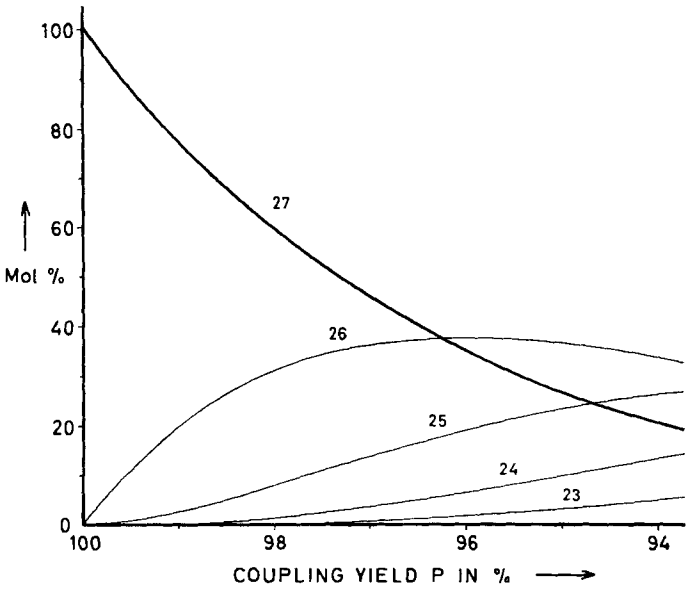

Fig. 1. *Sequential synthesis of secretin, a heptacosapeptide. Relative statistical distribution of peptides after 26 coupling steps, as a function of a constant coupling yield* P *in each step.* The curves marked 26, 25, etc., represent the total mol-percentage of all possible peptides containing 26, 25, etc., residues

99.8% of the amino component could be established, which however, need not imply a coupling yield of 99.8–99.9%. With such monitoring the REMA-synthesis of secretin seemed feasible, at least with regard to failure sequences; the problem of truncated peptides will be dealt with later.

In the REMA-synthesis, starting from the C-terminal hexadecapeptide, secretin 12–27 [10], *a priori* the following difficulties were expected: a) couplings gradually proceding more slowly; b) a possible Asp(3)-Gly(4) ($\alpha \rightarrow \beta$)-rearrangement; c) side-reactions during the coupling of histidine, the N-terminal amino acid residue.

a) In view of the supposed decreasing solubility of the growing amino component the coupling times were expected to increase. Since this increases the risk of by-products, attempts were made to limit the reaction time to that corresponding to removal of 99.8–99.9% of amino component. For this purpose the excess of acylating agent was increased (2-fold instead of 1.4-fold excess) [10]. Moreover, increase in concentration of the amino component to a maximum ($\geqslant 0.01$ mmol/ml) was attempted. – b) The (Asp(3)-Gly(4))-rearrangement [17] was investigated with the aid of a model peptide [18], and conditions were found for which this rearrangement was minimal or nil; these were applied in the synthesis of secretin. – c) In a preliminary synthesis of secretin [9] the excess mixed anhydride coupling of the N-terminal was avoided by utilizing histidine in the form of its *p*-nitrophenyl ester according to *Bodanszky et al.* [19]. In order to achieve the all-REMA synthesis now reported, the excess mixed anhydride coupling of histidine derivatives was investigated. The principal results for which the experimental data have been reported separately [20] are: protection of the imidazolyl moiety with the Z, the Boc, Bu^tOCO, and Tos groups resp. was highly satisfactory with regard to yield and racemization. The following groups were found to be unsuitable, Bzl, Dnp, and Ztf; imidazole-unprotected histidine was also unsuitable. Since in the REMA-synthesis of the secretin-sequence 2–27 invariably side-chain protecting groups to be removed hydrogenolytically were introduced, histidine in the form of its [N(α), N[imid.)-di-Z]-derivative, Z-His(Z), was used. All protecting groups could then be removed in one operation by hydrogenolysis.

3. Procedure adopted. – Starting from the hexadecapeptide **16** (seq. 12–27) [10], the completely protected secretin was synthesized; detailed stepwise lengthening of the polypeptide chain is given in *Scheme 2*.

As N-protecting group the Boc-group was used throughout except for histidine; previously we had also used the Z group [10]. Deprotection was effected by TFA[2]) solvolysis. The choice of protecting groups was such that intermediate products could be compared as far as possible with those synthesized by *Bodanszky et al.* [19] (see Table 1). For this reason the side-chains of serine, glutamic acid, and aspartic acid were protected with the Bzl group; Thr(5) and Thr(7) were also protected with the Bzl group, in contrast to the method described in [19] to avoid possible acylation of the hydroxyl group.

In the preliminary synthesis [9] the N-terminal histidine was coupled as the *p*-nitrophenyl ester [19]; in the main synthesis ((1–2)-mmol scale) it was directly coupled. The progress of the coupling reaction was monitored using fluorescamine (see below).

Scheme 2. *Detailed procedure for synthesis of secretin*

Deprotection	N_α-Protection		Sequence		Side-chain protection	Yield[a]	
H_2/Pd	Z	1	His		Z	80[b]	(94)
	Boc	2	Ser		Bzl	94	(96)
	Boc	3	Asp		Bzl	94	(97)
	Boc	4	Gly			91	(95)
	Boc	5	Thr		Bzl	96	(97)
	Boc	6	Phe			92	(95)
	Boc	7	Thr		Bzl	96	(100)
	Boc	8	Ser		Bzl	86	(97)
TFA	Boc	9	Glu		Bzl	94	(100)
	Boc	10	Leu			97	(99)
	Boc	11	Ser		Bzl	93	(96)
	Boc	12	Arg		NO_2	97	
	Boc	13	Leu			93	
	Boc	14	Arg		NO_2	99	
	Boc	15	Asp		Bzl	85	
	Boc	16	Ser		Bzl	89	
	Z	17	Ala			93	
	Z	18	Arg		NO_2	94	
	Z	19	Leu			87	
2N HBr/HOAc	Z	20	Gln			98	
	Z	21	Arg		NO_2	93	
	Z	22	Leu			91	
	Z	23	Leu			89	
	Z	24	Gln			98	
TFA	Boc	25	Gly			97	
	Z	26	Leu			96	
2N HBr/HOAc	Z	27	Val			90	
				NH_2			

[a]) Calculated on preceding protected peptide; in parenthesis yields of the (1–2)-mmol scale synthesis. Yield of protected secretin 13(22)%; average yield 93(95)% per step.

[b]) Nitrophenyl ester coupling.

4. Synthesis. – The yields obtained in the preliminary synthesis and in that on a (1–2) mmol scale are given in *Scheme 2*. Protected secretin could be obtained, with isolation of the intermediates but without further purification, in a yield of 13% (based on the C-terminal amino acid, valine) on the assumption that the deblocking steps always proceded quantitatively; this amounts to an average yield of 93% per step (calculated on isolated product). In the synthesis on a (1–2) mmol scale the yields were higher (presumably in consequence of smaller losses in operations on a larger scale, for the latter the overall yield of crude protected secretin was 22%, which amounts to an average step-yield of 95%. The reagent fluorescamine [16] was invaluable for monitoring by means of chromatographic spot tests by which the disappearance of 99.8–99.9% of the growing amino component could be established.

The lower limit of detection was 10–20 pmol. The coupling times, such as they were observed with a concentration of amino component of 10 μmol/ml, are shown in Fig. 2a and 2b.

Up to and including the hexadecapeptide a 1.4-fold excess of mixed anhydride was used, after that a twofold excess. Although small differences in pH of the reaction

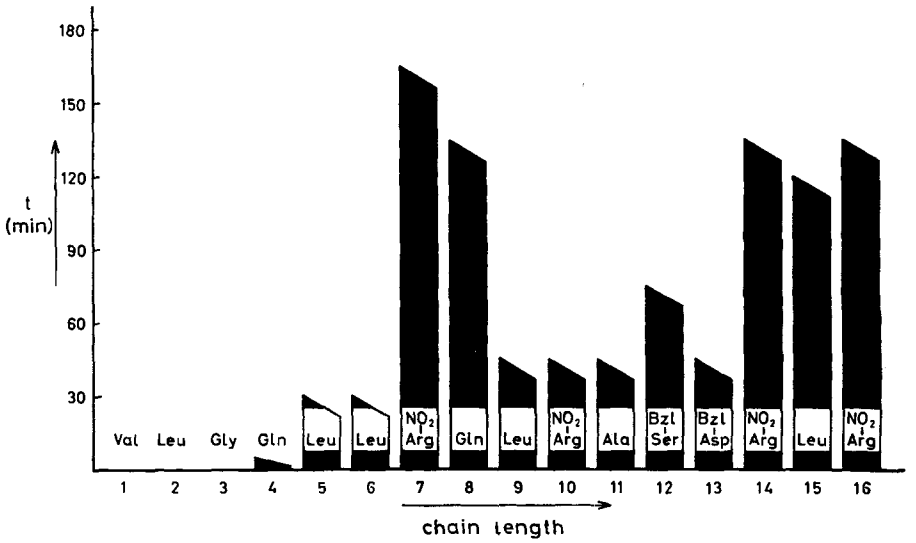

Fig. 2a. *Reaction times of couplings using REMA method*: disappearence of the amino component (c = 10 μmol/ml) > 99.9%; 1.4-fold excess of mixed anhydride

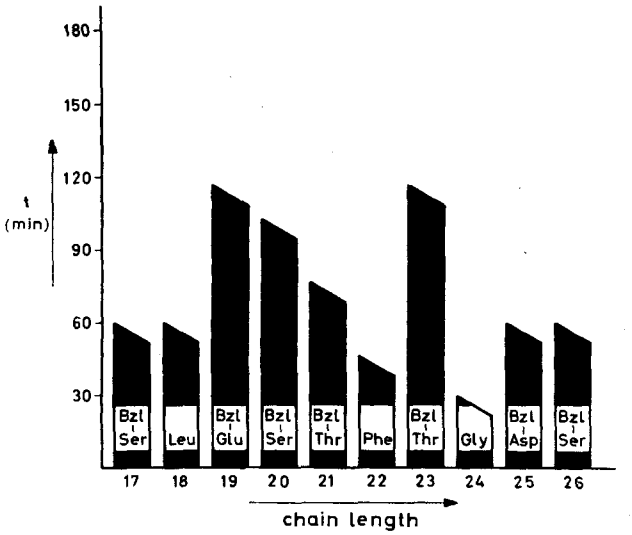

Fig. 2b. *Reaction times of couplings using REMA method*: disappearance of the amino component > 99.8%; twofold excess of mixed anhydride; c = 10 μmol/ml

mixture slightly affects coupling times, notable conclusions can be drawn from these orienting block diagrams. Thus, in the coupling of Boc-Arg(NO)$_2$-OH with three different leucyl peptides, a hexapeptide (seq. 22–27), a nonapeptide (seq. 19–27), and a pentadecapeptide (seq. 13–27), no gradual increase of reaction time with growing chain length of the amino component is observed. It appears that the longest reaction time (about 3 h) for attaining a presumable coupling yield of 99.8–99.9% is still quite acceptable on practical grounds.

For all intermediate products the purity was tested by TLC. and amino acid analysis. The physical properties of the intermediates up to coupling of Ser(8) were compared with the values found in [19] (Table 1). From the amino acid analyses, and from TLC. after removal of the Boc group, it could be inferred that, from Glu(9) on, coupling on the undesirable side of the mixed anhydride leading to truncated peptides had occurred (see Fig. 3 where progressive synthesis is shown). It is probable that this side reaction occurred to some extent in the coupling of each of the Thr(Bzl)(5) and (7) and in that of Phe(6) to the Thr(Bzl)-peptide (seq. 7–27). Also with Z-His(Z)-OH,

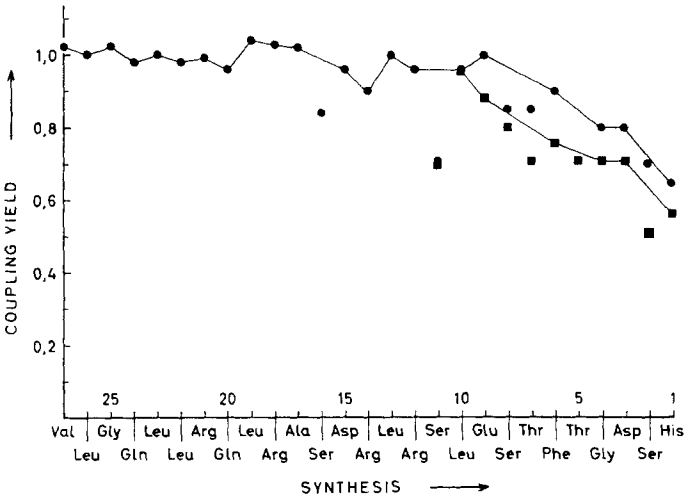

Fig. 3. *Incorporation of amino acids in the REMA-synthesis of secretin* (preliminary synthesis ■; main synthesis ●)

a decrease of incorporation was established; since this was the case in both the *p*-nitrophenyl ester and in the excess mixed anhydride method, transacylation of the Z group must be allowed for here too.

The difference in basicity between secretin, after removal of the N-protecting group, and any truncated peptide is particularly pronounced owing to the N-terminal His of secretin. Removal of the by-products as late as at the final stage appeared logical and was actually found to be possible simply by means of ion-exchange chromatography.

5. Deprotection and purification. – The protecting groups, *viz.* Z, Bzl, and nitro, were all removed in one operation by catalytic hydrogenolysis; the catalyst Pd/C(10%) was used in relatively large amounts and the reaction was carried out in 80% aqueous acetic acid for 40 h at room temperature. These conditions entail the

risk that Phe might be hydrogenated to CHA[2]), such hydrogenation of Phe having been observed by *Young et al.* in their synthesis of bradykinin [21]. The presence of CHA in REMA-secretin was investigated, and it was found that CHA had probably been formed, but to an extent of not more than 3%. After TLC. in ten systems, and paper-electrophoresis at high voltage, crude unprotected REMA-secretin showed only one spot which was intensively fluorescamine- and ninhydrin-positive. However, contaminating truncated peptides could be detected by the *Reindel-Hoppe* reagent after chlorination. A suitable purification method is the counter-current distribution as applied by *Jorpes & Mutt* [22] and by *Bodansky et al.* [19] for the purification of natural and synthetic secretin, respectively. It had been found, however, that depending on the solvent system a decrease of biological activity may occur [19]. The ion-exchange chromatographic method which appeares preferable had been successfully applied by *Wünsch et al.* for purification of their synthetic secretin [23]. No decrease of biological activity could be observed, it was eminently suitable for the removal of the truncated peptides. We used SP-Sephadex C-25 and block buffers of ammonium hydrogen carbonate at temperatures 0–5° to avoid troublesome evolution of carbon dioxide in the column (see Fig. 4)[4]). The loss of material during purification was about 50%; actually this value is comparatively low as crude REMA-secretin contained about 40% of contaminating truncated sequences as evaluated by the amino acid incorporation (Fig. 3). The yield of purified secretin, calculated on the basis of the C-terminal valine residue, amounted to about 5%. UV.-detection of products, using 206 and 254 nm simultaneously, is shown in Fig. 4, from which the absence of Bzl and NO_2 groups in the main product could also be deduced.

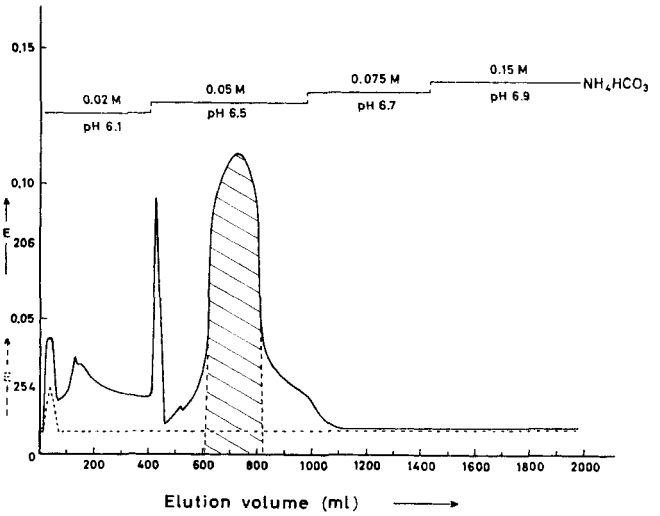

Fig. 4. *Ion-exchange chromatography*, SP-Sephadex C-25; full line 206 nm, dotted line 254 nm

6. Characterization and biological activity. – After acid hydrolysis, as well as after enzymatic hydrolysis by aminopeptidase-M (AP-M), the amino acid composition of purified REMA-secretin was in agreement with the theory (Table 2);

[4]) Ammonium acetate block buffers were not satisfactory.

Table 1. *Comparison of the physical properties of secretin sequences*

Sequence [a]	Optical rotation ($c = 2$, HOAc)					M.p. (dec.) (°C)		% N		
	REMA			[19]		REMA	[19]	Calc.	REMA found	[19] found
	$[\alpha]_D$ [b]	$[\alpha]_{546}$ [b]	$[\alpha]_{436}$ [b]	$[\alpha]_D$	$[\alpha]_{436}$					
16	−29.5	−36.5	−74.5	−30		~230	240	20.8	20.4	20.4
17	−26	−32	−66	−27		~300	240	19.9	19.6	19.4
18	−18	−29	−60	−20		~300	305	19.6	20.0	19.6
19	−19	−28	−57	−23	−57	~310	315	18.6	17.4	18.4
20	−18	−22	−51	−18	−52	~305	320	18.0	17.8	18.0

[a] Symbols refer to Table 4 (REMA synthesis).
[b] All temperatures between 20 and 30°, in accordance with those in [19], [α] values in degrees.

Table 2. *Amino acid analyses of purified REMA-secretin*

	Asp	Thr	Ser	Gln	Glu	Gly	Ala	Val	Leu	Phe	His	Arg	Peptide content
Theory	2	2	4	2	1	2	1	1	6	1	1	4	
Acid hydrolysis I	1.98	1.82 [a]	3.50 [a]		3.10	2.02	1.08	1.04	6.08	0.85	0.96	3.97	70%
Acid hydrolysis II	1.97	1.78 [a]	3.44 [a]		3.01	1.98	1.06	1.04	6.09	0.86	0.94	3.92	70%
Acid hydrolysis III [c]	1.93	1.83 [a]	3.52 [a]		3.06	1.98	1.01	0.96	6.04	0.87 [d]	1.04	3.94	68%
(AP-M) hydrolysis	1.92	− [b]	− [b]	− [b]	0.97	1.96	1.06	1.06	6.10	0.86	1.00	3.90	−

[a] Not corrected.
[b] Present but could not be determined.
[c] Determined by Dr. *H. Hindriks, Organon International* b.v., Oss.
[d] CHA $\leqslant$ 0.03.

a minor peak indicated the presence of $\leqslant 3\%$ of CHA. From the AP-M hydrolysis it could further be inferred that no racemization, and no Asp(3)-Gly(4) ($\alpha \rightarrow \beta$)-rearrangement had occurred, and that the glutamic acid/glutamine ratio was correct. Using L-amino acid oxidase (LAO), no D-amino acids ($< 2\%$) could be detected in a hydrochloric acid hydrolysate. Calculated on molecular weight 3056, the peptide content of the lyophilized secretin (acetate, hydrate) from aqueous acetic acid was 70%.

Purified REMA-secretin was compared chromatographically with secretin synthesized by *Wünsch et al.* [23] by fragment condensation. The two preparations behaved identically in a variety of chromatographic systems and on paper high-voltage electrophoresis. Peptide mapping, after trypsin digestion, followed by paper chromatography and column chromatography also indicated complete identity (Fig. 5). The optical rotatory dispersion (ORD.) spectra of these secretins were found to be identical within the limits of error (Fig. 6); the depth of the trough at 233 nm agrees with the value found by *Bodanszky et al.* [24].

Assays of biological activity of REMA-secretin are summarized in Table 3. REMA-synthesized secretin has a biological activity identical with that of natural secretin.

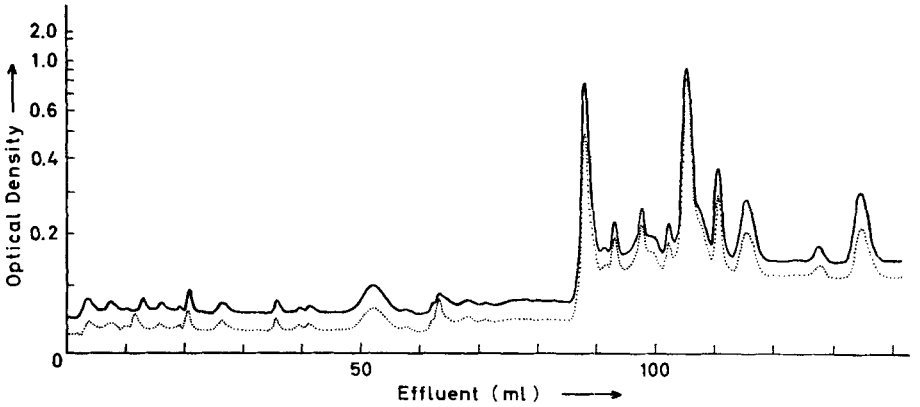

Fig. 5. *Separation of tryptic peptides on amino acid analyser*
···· REMA-synthesis (*A. van Zon*)
—— Fragment-synthesis (*E. Wünsch et al.*)

7. Conclusion and discussion. – The REMA-method was found to be highly suitable for the synthesis of secretin: a rate of chain lengthening by one amino acid per day could be attained on a 0.2 mmol-scale; difficulties sometimes experienced in the first stages of REMA-synthesis [5], owing to the water-solubility of small protected peptides did not occur; all intermediates could be isolated from the reaction mixture in high yields by precipitation with water. Neither on a 0.2 mmol-scale nor on a 2 mmol-scale the synthesis gave rise to any fundamental difficulties. As the peptide chain grew longer without increase in coupling times (see Fig. 2a and 2b), we conclude that the unknown conformation of the amino component is of greater importance

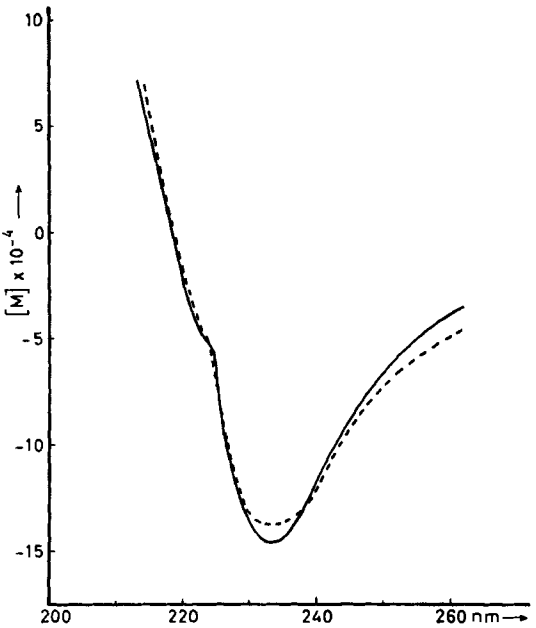

Fig. 6. *ORD. curves of secretin in water (pH 4.4)*
—— REMA-synthesis (*A. van Zon*)
- - - - Fragment-synthesis (*E. Wünsch et al.*)

Table 3. *Activity of REMA-secretin on pancreatic secretion (for methods see* [25])

Test animal	Assay	Activity [a] (clinical units/μg)	Investigator
rat	intact animal	3.3 [b]	*W. Y. Chey* (Rochester, N.Y.)
cat	isolated pancreas	3.1 (±0.15) [c] [d]	*T. Scratcherd* (Sheffield)
dog	intact animal	3.3 (2.7–4.0) [e]	*W. Y. Chey* (Rochester, N.Y.)
dog	intact animal	3.4 (2.9–4.3) [e]	*M. I. Grossman* (Los Angeles)

[a] For natural porcine secretin a biological activity of 4 clinical units/μg is assumed, see [25]; in all assays *Jorpes-Mutt* natural porcine secretin [22] was used as standard.
[b] A preliminary assay.
[c] In parenthesis limits of error.
[d] On standing at 4° in neutral solution 80% of the activity was lost within 3 h.
[e] In parenthesis, maximum possible range.

than its chain length as determining factor for coupling time. The yield of purified secretin, calculated on the basis of the C-terminal valine residue, amounted to about 5%.

By this synthesis the size-limit of the solid-phase method [11], about twelve amino acid residues for a purifiable peptide according to some authors [12], is amply exceeded. The REMA-method has the advantage that the quality of the intermediates can always be checked, and the couplings monitored. An assertion [26] that the mixed anhydride method would not be suitable for repetitive couplings owing to, *inter alia*, anomalous coupling, is largely refuted by our results. However, we feel that the size-limit of a purifiable peptide that can be synthesized by the REMA-

method has been reached at present, a limit which is of about thirty amino acid residues. Further development of the method, especially for avoidance of anomalous coupling and for attainment of even higher constant coupling yields is needed to effect synthesis of considerably larger peptides.

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Experimental part

M.p.'s were determined with *Anschütz* thermometers (samples in glass capillaries in a copper block). Optical rotations were measured on a *Perkin-Elmer* P-141 photoelectric polarimeter in a (1 dm)-cell and are estimated to be correct to $\pm 1°$. Elemental analyses were performed by Messrs. *M. van Leeuwen* and *H. M. A. Buurmans*. Amino acid analyses were carried out by Mr. *A. van Estrik*: samples were hydrolyzed with 6M HCl at 110° for 24 h and run on a *Locarte* (bench model) automatic amino acid analyser, norleucine was used as an internal standard. ORD. spectra were recorded by Mr. *J. C. Steeneveld* using a Spectropol I spectropolarimeter (*Fica*, Le Mesnil Saint-Denis, France).

Paper-electrophoresis was carried out on *Whatman* grade I paper, at 1300 V/40 mA, at about –5°, in formic acid/acetic acid/water 1:2:30 (pH 2) with a Pherograph, Type mini 65. TLC. was performed on silicagel plates (*Merck* 254) or on cellulose plates (*Schleicher & Schüll*) in the following systems (composition by volume): A) chloroform/methanol 4:1; B) butanol/pyridine/acetic acid/water 30:20:6:24; C) isopropyl alcohol/ethyl acetate/water 4:3:3; D) 2-methyl-2-butanol/isopropyl alcohol/water 32:32:36; E) chloroform/methanol/17% ammonia 2:2:1; F) butanol/acetic acid/water 4:1:1; G) butanol/pyridine/acetic acid/water 60:20:6:24; H) butanol/acetic acid/water 40:11:50; I) butanol/pyridine/water 40:10:16; J) ethyl acetate/acetic acid/water 15:15:10; K) butanol/pyridine/water 1:1:1. Spots were detected by UV. light, or by spraying with ninhydrin, or fluorescamine (detection at about 360 nm), or by *Reindel-Hoppe* reagent after chlorination. The Rf-values were often rather variable and should be regarded as merely a general indication of chromatographic behaviour. Dr. *J. M. A. Baas* wrote the computer program for the peptide-distribution graph (Fig. 1).

Most protected amino acids were purchased from the *Protein Research Foundation*, Osaka, Japan, *E. Merck AG*, Darmstadt, West Germany, and *Fluka AG*, Buchs, Switzerland. The purity of all commercial preparations was checked. M.p.'s, optical rotations, and TLC.-behaviour of synthetic products were found to be in agreement with literature data.

REMA-synthesis. – For the synthesis of protected secretin (*cf. Scheme 1*), the coupling reactions (*cf.* Fig. 2a and 2b) were carried out according to the following general procedure. The physical properties of secretin sequences are compared with those of [19] in Table 1. Yields, physical properties and analytical results are summarised in Table 4, 5, and 6.

General procedure. This differed only in one respect from that described in [10]: instead of a 1.4-fold excess, a 2-fold excess of reagent was used, *e.g.* 2.0 equiv. of isobutyl chloroformate were added to a solution of 2.2 equiv. of N-protected amino acid and 2.2 equiv. of NMM in DMF at a temperature of –15°.

Table 4. *REMA-synthesis* [a])

Sequence	Mixed anhydride							Amino component [b])				Yield [c])		
	Protected amino acid			NMM	iso-BuOCOCl		DMF				DMF			
		mg	μmol	μl	μl	μmol	ml		mg	μmol	ml	mg	μmol	%
17 Boc-Ser(Bzl)-S [d])	Boc-Ser(Bzl)	118	400	45	48	365	3	Boc-S [b])	420	178	7	418	165	93(96)
18 Boc-Leu-Ser(Bzl)-S	Boc-Leu · H_2O	85	340	38	41	310	3	**17**	390	154	7	396	150	97(99)
19 Boc-Glu(Bzl)-Leu-Ser(Bzl)-S	Boc-Glu(Bzl) [e])		310	35	37	280	7	**18**	372	140	7	379	132	94(100)
20 Boc-Ser(Bzl)-Glu(Bzl)-Leu-Ser(Bzl)-S	Boc-Ser(Bzl)	80	271	30	33	246	5	**19**	352	122	7	322	106	86(97)
21 Boc-Thr(Bzl)-Ser(Bzl)-Glu(Bzl)-Leu-Ser(Bzl)-S	Boc-Thr(Bzl)	68	218	24	26	198	3	**20**	300	99	7	306	95	96(100)
22 Boc-Phe-Thr(Bzl)-Ser(Bzl)-Glu(Bzl)-Leu-Ser(Bzl)-S	Boc-Phe	51	190	21	23	170	3	**21**	277	86	6	268	79	92(95)
23 Boc-Thr(Bzl)-Phe-Thr(Bzl)-Ser(Bzl)-Glu(Bzl)-Leu-Ser(Bzl)-S	Boc-Thr(Bzl)	50	160	18	19	145	3	**22**	245	72	4	247	69	96(97)
24 Boc-Gly-Thr(Bzl)-Phe-Thr(Bzl)-Ser(Bzl)-Glu(Bzl)-Leu-Ser(Bzl)-S	Boc-Gly	27	150	17	17	130	3	**23**	238	67	4	220	61	91(95)
25 Boc-Asp(Bzl)-Gly-Thr(Bzl)-Phe-Thr(Bzl)-Ser(Bzl)-Glu(Bzl)-Leu-Ser(Bzl)-S	Boc-Asp(Bzl)	42	130	14	15	117	2	**24**	212	59	4	210	55	94(97)
26 Boc-Ser(Bzl)-Asp(Bzl)-Gly-Thr(Bzl)-Phe-Thr(Bzl)-Ser(Bzl)-Glu(Bzl)-Leu-Ser(Bzl)-S	Boc-Ser(Bzl)	36	120	14	14	106	2	**25**	202	53	3.5	200	50	94(96)
27 Z-His(Z)-Ser(Bzl)-Asp(Bzl)-Gly-Thr(Bzl)-Phe-Thr(Bzl)-Ser(Bzl)-Glu(Bzl)-Leu-Ser-(Bzl)-S	Z-His(Z)ONp [f])	114	210					**26**	194	48	1	178	40	83(94)

a) Excess mixed anhydride couplings were carried out according to general procedure (see exper. part).
b) Boc groups were removed by TFA.
c) Yield calculated on preceding protected peptide; in parenthesis yields of the (1–2)mmol-scale synthesis.
d) S = -Arg(NO_2)-Leu-Arg(NO_2)-Asp(Bzl)-Ser(Bzl)-Ala-Arg(NO_2)-Leu-Gln-Arg(NO_2)-Leu-Leu-Gln-Gly-Leu-Val-NH_2 prepared according to [10].
e) Boc-Glu(Bzl) was liberated from its DCHA-salt.
f) Excess mixed anhydride coupling of Z-His(Z) in the (1–2) mmol-scale synthesis.

Table 5. *Physical properties and analytical results of intermediate products*

Sequence[a]	M.p. (dec)	$[\alpha]_D$[b]	$[\alpha]_{546}$[b]	Thin layer chromatography						Yield (%) [c]
				Rf^A	Rf^B	Rf^C	Rf^D	Rf^E	Rf^F	
16	~230	−29.5	−36.5	0.05	0.77	0.80	0.68	0.95		31[d])
17	~300	−26	−32	0	0.78		0.62	0.95		93 (96)
18	~300	−18	−29	0.05	0.77	0.70	0.63	0.95	0.58	97 (99)
19	~310	−19	−28	0.05	0.77	0.76	0.65	0.95	0.59	94 (100)
20	~305	−18	−22	0.05	0.77	0.83	0.64	0.95	0.28	86 (97)
21	~310	−17	−23	0.15	0.77	0.85	0.66	0.95	0.52	96 (100)
22	~320			0.28	0.82	0.94	0.73		0.73	92 (95)
23	~320			0.31	0.79	0.90	0.73		0.72	96 (97)
24	~310			0.25	0.82	0.92	0.73		0.77	91 (95)
25	~310			0.10	0.81	0.89	0.68		0.62	94 (97)
26	~325			0.10	0.81	0.89	0.68		0.62	94 (96)
27	~310			0.12	0.82	0.82	0.66		0.65	83[e]) (94)
				Overall yield seq. 1–27						13 (22)

a) Symbols refer to Table 4 (REMA-synthesis).
b) $[\alpha]$ values in degrees, $c = 2$, HOAc.
c) Yield based on preceding protected peptide; in parenthesis yields of the (1–2) mmol-scale synthesis.
d) Overall yield.
e) Nitrophenyl ester coupling according to [19].

For the following particular operations only representative examples are given.

Removal of protecting groups. The fully protected heptacosapeptide amide (160 mg) was dissolved in 60 ml of 80% acetic acid (prehydrogenated) and hydrogenolysed for 40 h at room temp. and atmospheric pressure after the addition of 250 mg of Pd/C (10%) catalyst. The solution was evaporated *in vacuo* at room temp. and the residue dissolved in 50 ml of 1% acetic acid and lyophilized, yielding 124 mg of crude secretin. Analysis: Asp 1.68, Thr 1.34, Ser 2.94, Glu 2.84, Gly 1.78, Ala 1.11, Val 1.07, Leu 5.98, Phe 0.61, His 0.69, Arg 3.78.

Purification of crude secretin was achieved by ion-exchange chromatography on SP-Sephadex C-25 mainly according to the procedure of *Wünsch et al.* [23]. On an equilibrated 27 cm column (diameter 0.9 cm) 64 mg of crude secretin was eluted upwards at about 5° (speed 20 ml/h) with successively a) 0.02M NH_4HCO_3, pH 6.1, 380 ml; b) 0.05M NH_4HCO_3, pH 6.5, 600 ml; c) 0.075M NH_4HCO_3, pH 6.7, 460 ml; d) 0.15M NH_4HCO_3, pH 6.9, 380 ml. For detection *Uvicord* III (206 and 254 nm) was used (see Fig. 4). The fractions of the principal peak were pooled and yielded, after three lyophilizations from 1% aqueous acetic acid, 27 mg of purified secretin (peptide content 70%, calc. on mol.wt. 3056).

Purified REMA-secretin displayed the same chromatographic behaviour as *Wünsch's* secretin in the systems A–D and F–K, and on high-voltage electrophoresis. Amino acid analyses are given in Table 2.

Enzymatic digestion. a) With aminopeptidase M (AP-M). To 100 nmol of secretin, dissolved in 250 µl of 0.1M 'Trisbuffer' (pH 7.8), a solution of 0.1 mg of AP-M (5000 mUnits/mg; *Röhm*, Darmstadt, West Germany) and 5 µl of toluene were added, and the solution left at 37° for 24 h. The amino acid analysis gave results identical with those obtained from acid hydrolysates, within the limits of error.

b) With (L-amino acid)-oxidase. To hydrolysed secretin (60 nmol) dissolved in 200 µl of 0.2M 'Trisbuffer' (pH 7.2–7.5) 10 µl of a solution of (L-amino acid)-oxidase (*Crotalus Adamanteus*; 7.97 mg/ml; 6.8 units/mg Prot; from *Sigma Chemical Company*, St. Louis, USA) and 5 µl of toluene were added, and the mixture shaken in an oxygen atmosphere at 37° for 24 h. In this case no significant racemization within the limits of error (2%) for Ala, Val, Leu, Phe, His, and Arg could

Table 6. *Amino acid analyses of secretin sequences*

Sequence[a]	Amino acid analysis										
	Asp	Thr[b]	Ser[b]	Glu	Gly	Ala	Val	Leu	Phe	His	Arg
16	0.84 (1)		0.85 (1)	1.93 (2)	1.08 (1)	1.13 (1)	1.06 (1)	5.22 (5)			3.72 (4)
17	0.92 (1)		1.58 (2)	1.99 (2)	1.03 (1)	1.04 (1)	1.01 (1)	4.92 (5)			3.57 (4)
18	0.92 (1)		1.46 (2)	2.01 (2)	1.04 (1)	1.04 (1)	1.00 (1)	5.92 (6)			3.57 (4)
19	0.83 (1)		1.47 (2)	3.12 (3)	1.03 (1)	1.05 (1)	1.00 (1)	5.80 (6)			3.44 (4)
20	0.97 (1)		2.26 (3)	2.81 (3)	1.04 (1)	1.07 (1)	1.08 (1)	5.99 (6)			3.77 (4)
21	0.97 (1)	0.97 (1)	2.28 (3)	2.78 (3)	1.10 (1)	1.11 (1)	1.07 (1)	5.94 (6)			3.76 (4)
22	0.95 (1)	0.69 (1)	2.21 (3)	2.80 (3)	1.09 (1)	1.10 (1)	1.08 (1)	6.13 (6)	0.84 (1)		3.83 (4)
23	1.01 (1)	1.68 (2)	2.38 (3)	2.80 (3)	1.11 (1)	1.10 (1)	1.04 (1)	5.95 (6)	0.75 (1)		3.76 (4)
24	1.00 (1)	1.41 (2)	2.41 (3)	2.83 (3)	1.83 (2)	1.08 (1)	1.06 (1)	6.04 (6)	0.77 (1)		3.79 (4)
25	1.82 (2)	1.38 (2)	2.35 (3)	2.85 (3)	1.80 (2)	1.11 (1)	1.00 (1)	6.04 (6)	0.75 (1)		3.80 (4)
26	1.66 (2)	1.36 (2)	3.08 (4)	2.75 (3)	1.79 (2)	1.10 (1)	1.07 (1)	6.08 (6)	0.74 (1)		3.85 (4)
26[c]	1.86 (2)	1.13 (2)	1.57 (4)	2.82 (3)	1.79 (2)	1.08 (1)	1.07 (1)	6.03 (6)	0.74 (1)		3.86 (4)
27	1.47 (2)	1.30 (2)	2.81 (4)	2.77 (3)	1.77 (2)	1.10 (1)	1.06 (1)	6.07 (6)	0.70 (1)	0.55 (1)	3.75 (4)
27[c]	1.72 (2)	1.03 (2)	1.27 (4)	2.76 (3)	1.80 (2)	1.11 (1)	1.07 (1)	6.06 (6)	0.69 (1)	0.54 (1)	3.81 (4)

[a] Symbols refer to Table 4 (REMA synthesis).
[b] Not corrected.
[c] Hydrolysed with (HOAc/12M HCl) 1:1 at 110° for 24 h.

be detected; racemization of Ser, Thr, Asp, and Glu could not be determined since these amino acids are digested very slowly by (L-amino acid)-oxidase, if at all.

c) *Tryptic digestion.* To 50 nmol of secretin dissolved in 1 ml of 1% ammonium hydrogen carbonate, 60 µl of a trypsin (*Boehringer*, Mannheim, West Germany) solution (2 mg of trypsin/ml of 1% NH_4HCO_3) were added. After standing for 4 h at room temp. a further 30 µl of trypsin solution was added, 1 h later the mixture was lyophilized, and the residue was dissolved in 250 µl of hot water. Peptide mapping was effected by means of paper chromatography (descending on *Whatman* no. 1, in butanol/pyridine/acetic acid/water 30:20:6:24) and use of an amino acid analyser; for results see Fig. 5.

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