respect the trichloromethyl ethers appear to be unique. More precisely whatever effect there may be is usually smaller than or at most comparable in magnitude with the effects of intermolecular field-gradients and of other intramolecular interactions. This perhaps casts doubt on the mechanism which has been proposed in [1] to explain the marked frequency variation of the α-chloroethers and [2] the difference between *cis-* and *trans*-1,2-dichloroacenaphthylene. In this respect the frequencies of the trichloromethyl group in compounds of the type $Cl_3C\ (CH_2)_n\ CH_2Cl$ are of interest [10] for sufficiently long chain-lengths (n = 8, 10, 12) the resonances fall into two groups: one resonance at 38.6 MHz and two others very close together at 37.3 MHz. This is in contrast to the situation with short chain lengths where, despite the proximity of the CH_2Cl group, the resonances of the CCl_3 group spread over only 0.6–0.7 MHz. This observation is likewise in agreement with the results presented [2] for the variously oriented 1,2-dichloro compounds. The frequency variations which have been observed may thus well be due to stronger sigma-sigma interactions between vicinal C–Cl and C–C bonds than between two vicinal C–Cl bonds.

In conclusion this survey of ^{35}Cl resonances of the trichloromethyl group shows that with most planar conjugating groups the change in frequency with orientation is less than of 1.0 MHz, far less than has been clearly demonstrated for the α-chloro ethers. When the trichloromethyl group is attached to a tetracoordinated carbon atom, shifts of at least the same order of magnitude can occur.

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173. Repetitive Excess Mixed Anhydride (REMA) Synthesis of Peptides. The Protected C-terminal Hexadecapeptide of Secretin

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(30. IV. 73)

Summary. The usefulness of the repetitive excess mixed anhydride (REMA) method of peptide synthesis was demonstrated by the prompt synthesis of the protected C-terminal hexadecapeptide of the gastrointestinal hormone secretin, Boc-Arg(NO_2)-Leu-Arg(NO_2)-Asp(Bzl)-

[1]) Taken in part from the Doctoral Dissertation to be submitted by *A. van Zon*.

Ser(Bzl)-Ala-Arg(NO_2)-Leu-Gln-Arg(NO_2)-Leu-Leu-Gln-Gly-Leu-Val-NH_2 (secretin 12–27). The overall yield was 31%, with an average coupling yield of 93%.

Comparison of the physical constants of all intermediate sequences with those published, by *Bodanszky et al.* and by *Wünsch et al.* in their syntheses of secretin, indicated identity. No significant racemization could be detected in acid hydrolysates on digestion with L-amino acid oxidase.

Introduction. – The sequential synthesis of peptides by what we call the REMA method (Repetitive Excess Mixed Anhydride Method) [1] is based on the use of mixed anhydrides of the carbonates [2].

In the period when mixed anhydrides were first used racemization appeared to be serious, but this difficulty could be overcome by finding the conditions for racemization-free coupling [3]. *Weygand et al.* then made use of an excess of symmetrical anhydrides – sometimes not readily accessible –, a procedure by which reactions were forced to proceed to completion, while coupling on the undesirable side of the mixed anhydride (a side-reaction to which we will revert later) was evaded [4]. The principle of excess is essential to obtain near-quantitative coupling [5], otherwise sequential syntheses will result in deleted sequences. *Tilak et al.*, when using excess mixed anhydrides, found that the excess could be promptly destroyed by aqueous potassium hydrogen carbonate [6]. The yields with small peptides were so high that the method could be applied repetitively without purification of the intermediate products.

Tilak et al. succeeded in producing a nonapeptide in this way [6]. In our Laboratory the sequence 1–10 of human growth hormone (HGH 1–10) was synthesized by the REMA method [1] [7]; the product, on deprotection, was found to be identical with the corresponding peptide synthesized by the solid-phase method [8]. The problem of reaction on the undesirable side of the mixed anhydride, which results in carbamates, *e.g.* in the couplings to N-terminal proline peptides, was evaded by the coupling of dipeptides and larger peptides.

One of us has discussed a comparison of the highly satisfactory REMA method [7] with *Merrifield*'s solid-phase peptide synthesis [9]. A salient point still to be investigated was the extension limit with respect to the chain length. It may be recalled that by some authors the present limit of the solid-phase synthesis is considered to be a chain length of about 12–15 amino acid residues, for a purifiable peptide [10].

Objective. – We started such an investigation, choosing as model the gastrointestinal hormone secretin, which with 27 amino acid residues is approximately three times larger than the HGH 1–10 which was readily synthesized here.

Secretin was already synthesized stepwise by *Bodanszky et al.* by means of the active ester method [11], so that, if the same protecting groups are used, the physical constants of the sequences synthesized by the REMA method can be compared with the data from the literature. For a number of small sequences the physical constants given by *Wünsch et al.* [12] in their synthesis of secretin can also be involved in the comparison.

We now wish to report the synthesis of the C-terminal hexadecapeptide sequence of secretin, with which the previously mentioned limit of the solid-phase synthesis was attained.

Secretin. – Secretin was discovered as far back as 1902 by *Bayliss & Starling* [13] in the mucous membrane of the duodenum, but it was not until 1961 that the attempt to isolate the peptide hormone in the pure state was successful [14]. For the isolation of 10 mg of hormone with an activity of 4000 clinical units per mg 10,000 hogs were required. Finally the primary structure of secretin was elucidated in 1966 by *Jorpes & Mutt* [15]. The hormone is found to consist of 27 amino acid residues (molweight 3056). The sequence is as follows[2]) (Fig. 1):

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

Fig. 1. *The amino acid sequence of the (porcine) hormone Secretin*

The above sequence was corroborated by two syntheses of *Bodanszky, Ondetti et al.* [11], and more recently by the synthesis of *Wünsch et al.* [12]. In the elucidation of the sequence use was made of degradation with the aid of trypsin. This breakdown of secretin is so distinct that the chromatographic pattern of the resulting peptide fragments can serve as a criterion of purity.

The biological activity of secretin is as follows: (i) the pancreas is stimulated to release hydrogencarbonate, (ii) gastrin activity is inhibited, (iii) (iv) the secretion of bilious juices as well as of insulin is increased, (v) the motor activity of stomach and duodenum is decreased [17].

A striking feature is the great resemblance between the sequence of secretin from the duodenum and that of the hormone glucagon from the pancreas, although the biological activity of these compounds is different. The high degree of homology indicates that the two peptide hormones have the same ancestry.

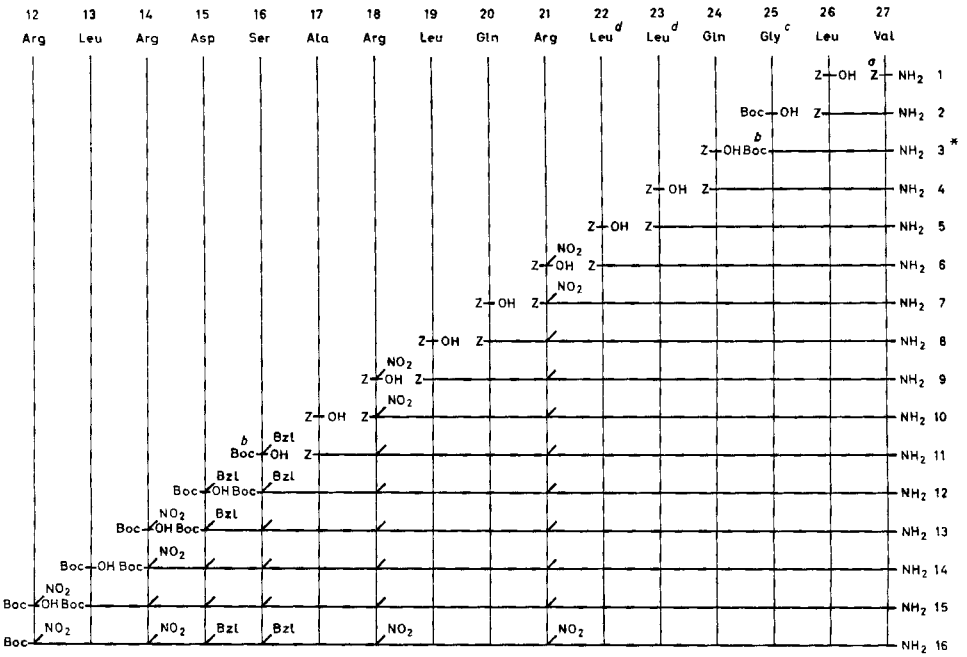
Results. – In our REMA synthesis of the C-terminal hexadecapeptide of secretin (secretin 12–27) both the benzyloxycarbonyl group (Z) and the *t*-butoxycarbonyl group (Boc) were used as α-amino protecting groups. The Z-group was removed by hydrogen bromide/acetic acid and the Boc-group by trifluoroacetic acid. The choice of Z- and Boc-groups was such that a comparison of the physical constants of the intermediate products with those of *Bodanszky* [11] and *Wünsch* [12] became possible. The same argument applied to the choice of the protecting groups in the side-chains, where Arg(NO_2) was used four times, and Ser(Bzl) and Asp(Bzl) once each (Scheme).

All the amino acids occurring in secretin 12–27 (Fig. 1), including Z-Gln, which might cause imide formation or dehydration, and Boc-Gly[3]), where diacylation appeared possible [6], could be coupled in high yields without any difficulty. It was only the coupling of Z-Arg(NO_2) and Boc-Arg(NO_2) which involved the formation

[2]) Standard abbreviations are used for amino acids and protecting groups [16]. In addition, DCHA stands for dicyclohexylamine, DMF for N,N'-dimethylformamide, DMSO for dimethylsulfoxide, NMM for N-methylmorpholine, TFA for trifluoroacetic acid, and TLC. for thin-layer chromatography.

[3]) Dr *K. Lübke*, Schering A.-G., Berlin, also observed no difficulty in the coupling of Boc-Gly by the REMA method (personal communication to *H. C. Beyerman*, in Boston, June 1972).

Scheme. *REMA Synthesis of the Protected C-Terminal Hexadecapeptide of Secretin (12–27)*



a) Z-groups were removed by acidolysis (2 N HBr/HOAc).
b) Deprotection by trifluoroacetic acid.
c) Gly was also incorporated by means of Z-Gly-ONp.
d) Leu was also coupled as the Boc-derivative.

of minor by-products, but these could be removed during working-up without causing any appreciable reduction of the yield. These by-products are probably produced upon lactam formation from the excess of the mixed anhydride of N-protected Arg(NO_2).

The process briefly described in the experimental part differs in one respect from the one described by one of us in a previous communication [7]. The couplings now were monitored by making use of fluorescamine, the new highly sensitive reagent [18]. Fluorescamine was found to be ten to a hundred times more sensitive than ninhydrin for the detection of unreacted amino groups, dependent on the amino component.

As a rule we continued a peptide coupling until $\geqslant 99.9\%$ of the amino component no longer reacted with fluorescamine, but this need not mean that the coupling proceeded to such a high percentage.

With isolation of the intermediate products by precipitation by means of the addition of water or aqueous sodium chloride, but without further purifications, the hexadecapeptide secretin 12–27 could be synthesized in an overall yield of 31%, which means an average coupling yield of 93% (calculated on Z-Val-OH, and assuming a quantitative yield in each deblocking step). All the intermediates gave a correct amino acid analysis and, after one crystallization, a good elemental analysis (Tables

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

Sequence[a]	Optical rotations ($c = 2$, HOAc)					M.p. (dec.)			Yields (%)	
	REMA		[11 b, c]	[12 a]		REMA	[11 b, c]	[12 a]	REMA	[11 b, c]
	$[\alpha]_D$[b]	$[\alpha]_{546}$[b]	$[\alpha]_D$	$[\alpha]_D$	$[\alpha]_{546}$					
1	+22[c]	+27	+22	+22.6	+26.9	205–8	206–8	212	90	77.5
1A[d]	+21[e]	+25		+22.4	+24.2	241–3	248–53	242	99	
2	−24	−29	−28	−24.4	−29.7	230–1	235–6	231–2	97	90
3	−29.5	−36	−30			185–6	186–7			86
3*	−32.5	−39.5				199–200			97	
4	−26.5	−32	−27	−24.6	−29.7	239–40	239–41	239.5–40.5	98	95
5	−34.5	−41.5	−35			260–1	270–2		89	93
5*	−19[c]	−23		−19.8	−23.5	231–3		233–5		
6	−45.5	−55	−47			259–61	264–7		91	100
6*	−30.5[c]	−36.5		−31.5	−37.8	252–4		255		
7	−38	−45	−40			239–40	252		93	92
8	−30[f]	−35.5	−32			249–50	250		98	100
9	−43	−51.5	−41			266–8	267–9		87	99
10	−35	−42.5	−36			257–9	255–64		94	88
11	−37.5[g]	−46	−38			272–4	258–64		93	95
12	−31[h]	−36	−33			~300	300		89	75
13	−26	−32	−27			~300	300		85	94
14	−34	−41	−39			~320	305		99	70
15	−32	−77[i]	−32, −76[i]			~250	245		93	98
16	−29.5	−36.5	−30			~230	240		97	
								Overall yield	31	

[a] Symbols refer to Scheme and Experimental Part.
[b] All temperatures between 20–30°, according to the literature.
[c] ($c = 1$, DMF). [d] HBr.H-Val-NH_2). [e] ($c = 2$, H_2O). [f] ($c = 2$, DMSO). [g] ($c = 2$, 80% HOAc). [h] ($c = 0.8$, 80% HOAc). [i] $[\alpha]_{436}$[b].

Table 2a. *REMA Synthesis*[a])

Sequence	Mixed anhydride								Amino component[b])				Yield[c])		
	Protected amino acid				NMM	*i*-BuOCOCl		DMF				DMF			
			g	mmol	ml	ml	mmol	ml		g	mmol	ml	g	mmol	%
Z-Val-NH_2	**1**	Z-Val	5.00	20.0	2.22	2.60	19.5	40[f])	NH_3(conc.)	10 ml		40[f])	4.40	17.6	90
Z-Leu-Val-NH_2	**2**	Z-Leu[e])		22.5	2.50	2.80	21.0	25[f])	**1**	3.80	15.2	30	5.30	14.6	96
Z-Gly-Leu-Val-NH_2[d])	**3**	Z-Gly-ONp	0.27	0.82					**2**	0.22	0.61	10	0.20	0.48	78
Boc-Gly-Leu-Val-NH_2	**3***	Boc-Gly	3.50	20.0	2.22	2.53	19.0	25	**2**	4.90	13.5	60	5.10	13.2	98
Z-Gln-Gly-Leu-Val-NH_2	**4a**	Z-Gln	1.20	4.3	0.48	0.53	4.0	15	**3**	1.20	2.85	10	1.54	2.80	98
Z-Gln-Gly-Leu-Val-NH_2	**4b**	Z-Gln	5.50	19.5	2.15	2.43	18.2	60	**3***	5.05	13.1	90	7.04	12.8	98
Z-Leu-Gln-Gly-Leu-Val-NH_2	**5**	Z-Leu[e])		5.5	0.61	0.68	5.1	15	**4b**	2.00	3.65	45	2.14	3.23	89
Boc-Leu-Gln-Gly-Leu-Val-NH_2	**5***	Boc-Leu · H_2O	4.50	18.0	2.00	2.24	16.8	40	**4b**	6.60	12.0	100	6.80	10.8	90
Z-Leu-Leu-Gln-Gly-Leu-Val-NH_2	**6**	Z-Leu[e])		3.4	0.38	0.42	3.2	15	**5**	1.46	2.20	55	1.55	2.00	91
Boc-Leu-Leu-Gln-Gly-Leu-Val-NH_2	**6***	Boc-Leu · H_2O	4.00	16.1	1.79	2.00	15.0	40	**5***	6.70	10.7	120	7.50	10.1	94
Z-Arg(NO_2)-Leu-Leu-Gln-Gly--Leu-Val-NH_2	**7**	Z-Arg(NO_2)	0.85	2.4	0.27	0.30	2.25	15	**6**	1.25	1.60	55	1.45	1.49	93
Z-Gln-Arg(NO_2)-Leu-Leu-Gln--Gly-Leu-Val-NH_2	**8**	Z-Gln	0.59	2.1	0.23	0.26	1.95	20	**7**	1.27	1.30	20	1.40	1.27	98
Z-Leu-Gln-Arg(NO_2)-Leu-Leu--Gln-Gly-Leu-Val-NH_2	**9**	Z-Leu[e])		1.5	0.17	0.19	1.4	10	**8**	1.10	1.00	25	1.05	0.87	87
Z-Arg(NO_2)-Leu-Gln-Arg(NO_2)-Leu--Leu-Gln-Gly-Leu-Val-NH_2 · H_2O	**10**	Z-Arg(NO_2)	0.29	0.83	0.09	0.10	0.78	10	**9**	0.67	0.55	20	0.74	0.52	94

a) Excess mixed anhydride couplings were carried out according to the General Procedure.
b) Z-groups were removed by 2N HBr/HOAc; Boc-groups by TFA.
c) Yield calculated on preceeding protected peptide.
d) Active ester coupling according to [11b].
e) Z-Leu was liberated from its DCHA-salt.
f) THF (ml).

Table 2b. *REMA Synthesis*[a])

Sequence		Mixed anhydride: Protected amino acid mg	μmol	NMM μl	*i*-BuOCOCl μl	μmol	DMF ml	Amino component[b])	mg	μmol	DMF ml	Yield[c]) mg	μmol	%
Z-Ala-Arg(NO_2)-Leu-Gln-Arg(NO_2)--Leu-Leu-Gln-Gly-Leu-Val-NH_2 · H_2O	**11**	Z-Ala	130	570	65	70	525	10	**10**	530	370	10	521	345	93
Boc-Ser(Bzl)-Ala-Arg(NO_2)-Leu-Gln--Arg(NO_2)-Leu-Leu-Gln-Gly-Leu-Val--NH_2	**12**	Boc-Ser(Bzl)	95	320	36	40	300	5	**11**	315	209	15	304	186	89
Boc-Asp(Bzl)-Ser(Bzl)-Ala-Arg(NO_2)--Leu-Gln-Arg(NO_2)-Leu-Leu-Gln-Gly--Leu-Val-NH_2	**13**	Boc-Asp(Bzl)	80	245	28	30	230	4	**12**	250	153	16	239	130	85
Boc-Arg(NO_2)-Asp(Bzl)-Ser(Bzl)-Ala--Arg(NO_2)-Leu-Gln-Arg(NO_2)-Leu-Leu--Gln-Gly-Leu-Val-NH_2	**14**	Boc-Arg(NO_2)	55	170	19	20	155	3	**13**	200	109	8	221	108	9[illegible]
Boc-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	**15**	Boc-Leu · H_2O	37	150	17	19	140	3	**14**	195	96	7	192	89	93
Boc-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 · H_2O	**16**	Boc-Arg(NO_2)	39	120	14	15	113	3	**15**	173	80	6	185	78	97

a) Excess mixed anhydride couplings were carried out according to the General Procedure.
b) Z-groups were removed by 2N HBr/HOAc; Boc-groups by TFA.
c) Yield calculated on preceeding protected peptide.

Table 3. *Physical properties and analytical results of intermediate products*

Sequence	Formula	M.p. (dec.)	$[\alpha]_D$[a] (c = 2, HOAc)	$[\alpha]_{546}$[a] (c = 2, HOAc)	% C calc.	% C found	% H calc.	% H found	% N calc.	% N found	Recrist.
1	$C_{13}H_{13}N_2O_3$	205–8	+22[b])	+27[b])	62.38	62.5	7.25	7.4	11.19	11.3	EtOH/H_2O
1A	$C_5H_{13}N_2OBr$	241–3	+21[c])	+25[c])	30.47	30.5	6.65	6.8	14.21	14.0	
2	$C_{19}H_{29}N_3O_4$	230–1	–24	–29	62.78	62.5	8.04	8.1	11.56	11.5	EtOH/H_2O
3	$C_{21}H_{32}N_4O_3$	185–6	–29.5	–36	59.98	60.0	7.67	7.6	13.33	13.5	H_2O
3*	$C_{18}H_{34}N_4O_5$	199–200	–32.5	–39.5	55.93	55.8	8.87	8.7	14.50	14.3	
4a	$C_{26}H_{40}N_6O_7$	238–9	–25	–30.5	56.92	56.7	7.35	7.4	15.32	15.3	MeOH/H_2O
4b	$C_{26}H_{40}N_6O_7$	239–40	–26.5	–32	56.92	56.9	7.35	7.2	15.32	15.4	MeOH/H_2O
5	$C_{32}H_{51}N_7O_8$	260–1	–34.5	–41.5	58.07	58.0	7.77	8.1	14.82	14.6	HOAc/H_2O
5*	$C_{29}H_{53}N_7O_8$	231–3	–19[b])	–23[b])	55.48	55.9	8.51	8.5	15.62	15.5	
6	$C_{38}H_{62}N_8O_9$	259–61	–45.5	–55	58.89	59.1	8.06	8.4	14.46	14.3	HOAc/H_2O
6*	$C_{35}H_{64}N_8O_9$	252–4	–30.5[b])	–36.5[b])	56.74	56.5	8.71	8.8	15.12	15.0	
7	$C_{44}H_{73}N_{13}O_{12}$	239–40	–38	–45	54.14	55.1	7.54	7.8	18.65	18.1	EtOH/H_2O
8	$C_{49}H_{81}N_{15}O_{14}$	249–50	–30[d])	–35.5[d])	53.29	53.6	7.39	7.7	19.03	18.7	EtOH/H_2O
9	$C_{55}H_{92}N_{16}O_{15}$	266–8	–43	–51.5	54.26	54.2	7.62	7.9	18.41	18.2	EtOH/H_2O
10	$C_{61}H_{105}N_{21}O_{19}$[e])	257–9	–35	–42.5	51.00	51.0	7.37	7.4	20.48	20.5	EtOH/EtOAc
11	$C_{64}H_{110}N_{22}O_{20}$[e])	272–4	–37.5[f])	–46[f])	50.98	51.0	7.35	7.7	20.44	20.4	EtOH/Et_2O
12	$C_{71}H_{121}N_{23}O_{21}$	~300	–31[g])	–36[g])					19.73	19.3	HOAc/H_2O
13	$C_{82}H_{132}N_{24}O_{24}$	~300	–26	–32					18.29	18.1	HOAc/H_2O
14	$C_{88}H_{143}N_{29}O_{27}$	~320	–34	–41					19.92	20.0	
15	$C_{94}H_{134}N_{30}O_{28}$	~250	–32	–39					19.52	19.7	
16	$C_{100}H_{167}N_{35}O_{32}$[e])	~230	–29.5	–36.5	50.64	50.2	7.10	7.2	20.67	20.4	% 0 calc. 21.59 found 21.7

[a]) All temperatures between 20–30°, according to the literature. [b]) (c = 1, DMF). [c]) (c = 2, H_2O). [d]) (c = 2, DMSO). [e]) Contains 1 mol of water according to O-analysis. [f]) (c = 2, 80% HOAc). [g]) (c = 0.8, 80% HOAc).

Table 4. *R_f-values and amino acid analyses of secretin sequences* (symbols refer to scheme and experimental part)

Sequence	TLC.					Amino acid analyses							
	R_f^A	R_f^B	R_f^C	R_f^D	R_f^E	Asp	Ser	Glu	Gly	Ala	Val	Leu	Arg
1	0.53	0.62											
2	0.55	0.64	0.76	0.87									
3	0.27	0.60	0.65	0.85					1.00(1)		0.99(1)	0.99(1)	
3*	0.24	0.50	0.42	0.86					1.00(1)		0.99(1)	0.99(1)	
4a	0	0.25	0.11	0.75				0.96(1)	1.04(1)		0.99(1)	1.01(1)	
4b	0	0.25	0.12	0.75				0.97(1)	1.02(1)		0.99(1)	1.02(1)	
5	0	0.29	0.24	0.79				0.92(1)	1.00(1)		1.01(1)	2.02(2)	
5*		0.29		0.94	0.82			0.99(1)	0.98(1)		1.00(1)	2.03(2)	
6	0	0.35	0.25	0.81				0.98(1)	1.00(1)		1.01(1)	2.98(3)	
6*		0.33		0.94	0.85			0.99(1)	0.99(1)		1.00(1)	3.00(3)	
7	0	0.15	0.05	0.74				0.99(1)	1.05(1)		1.04(1)	2.98(3)	0.95(1)
8	0	0.10	0.05	1				1.90(2)	1.01(1)		1.01(1)	3.02(3)	1.05(1)
9	0	0.10	0.05	1				1.86(2)	1.03(1)		1.02(1)	4.11(4)	0.97(1)
10	0	0.26	0	0.69				1.93(2)	1.03(1)		1.01(1)	3.96(4)	2.06(2)
11	0	0.05	0	0.63				1.90(2)	0.97(1)	1.03(1)	0.98(1)	4.02(4)	2.10(2)
12		0.05		1	0.76		0.84(1)	1.95(2)	0.99(1)	1.03(1)	1.00(1)	4.06(4)	1.98(2)
13		0.07		1	0.79	0.91(1)	0.91(1)	1.96(2)	1.02(1)	0.97(1)	1.00(1)	4.15(4)	1.97(2)
14		0.05		0.71	0.77	0.88(1)	0.74(1)	1.92(2)	1.03(1)	1.07(1)	1.05(1)	4.19(4)	2.87(3)
15		0.05		0.70	0.77	0.91(1)	0.85(1)	1.91(2)	1.04(1)	1.06(1)	1.03(1)	5.14(5)	2.93(3)
16		0.05	0	0.95	0.77	0.84(1)	0.85(1)	1.93(2)	1.08(1)	1.13(1)	1.06(1)	5.22(5)	3.72(4)

16 R_f (*i*-PrOH/EtOH/H_2O 4:3:3) 0.80; R_f (*t*-AmOH/*i*-PrOH/H_2O 32:32:36) 0.68.

3 and 4). The optical rotations and the melting points agree with the values given by *Bodanszky et al.* [11] and *Wünsch et al.* [12] (Table 1).

The synthesis was repeated on a larger scale; the results did not differ essentially from those of the first synthesis.

A sample of the hexadecapeptide was subjected, after acid hydrolysis, to digestion with L-amino acid oxidase; no significant racemization ($< 2\%$) could be detected.

Conclusions and discussion. – The repetitive excess mixed anhydride (REMA) method of coupling was found highly suitable for the synthesis of pure protected secretin 12–27. The synthesis proceeds relatively quickly, since about one amino acid a day can be added, and yields an unambiguous product without far-reaching purification. With this hexadecapeptide the limit of the solid-phase synthesis mentioned in the introduction has been equalled or exceeded. In addition the REMA method presents the advantage over the solid-phase synthesis that the quality of the intermediates can always be checked, while the couplings can be monitored by the use of the highly sensitive fluorescamine.

Difficulties which sometimes occur in the initial stage of a REMA synthesis, in consequence of the solubility in water of some small peptides, and which necessitate the use of extraction for the isolation [7], did not present themselves in the synthesis of secretin 12–27. All the intermediates could invariably be isolated by precipitation.

The coupling times of reactions at the same concentration, as they were deduced from spot tests with fluorescamine, were found to be less than 5 min for the preparation of small peptides, whilst for the addition to the pentadecapeptide nearly 2 h were required ($< 0.1\%$ of free amino component still present). The explanation of this increase, which was not a gradual one, may be a conformational change of the amino component.

As the solubility of the growing peptide chain decreases the reaction times may increase even further, *inter alia* owing to the necessary lower concentration. We hope to avoid an inconvenient increase of the reaction time by raising the concentration of the acylating agent.

We are continuing the REMA synthesis of secretin, but it appeared useful to us to communicate already now the result we have attained.

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Experimental Part

Melting points were determined with *Anschütz* thermometers with the samples contained in glass capillaries in a copper block. – Optical rotations were measured with 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 *M. A. Hoefnagel* of this Laboratory. – Amino acid analyses were carried out by Messrs *H. A. Billiet* and *C. Warnaar* of the Analytical Department and by Mr *A. van Estrik* of this Laboratory. Samples were hydrolyzed with 6M HCl at 110° for 24 h and run on a *Locarte* (bench model) automatic amino acid analyzer. – Thin-layer chromatography was performed on silicagel plates (*Merck* F254) in the following solvent systems

(composition by volume): A/Chloroform/acetic acid 9:1; B/Chloroform/methanol 4:1; C/Chloroform/methanol/acetic acid 85:10:5; D/Chloroform/methanol/17% ammonia 2:2:1; E/Butanol/pyridine/acetic acid/water 30:20:6:24. – Spots were visualized by UV. light, or on spraying with ninhydrin, or fluorescamine (detection at about 390 nm), or the *Reindel-Hoppe* reagent after chlorination.

Most protected amino acids were purchased from *Protein Research Foundation*, Osaka, Japan; *E. Merck A.G.*, Darmstadt, Germany, and *Fluka A.G.*, Buchs, Switzerland. Purity of all commercial preparations was checked. Melting points, optical rotations, and TLC.-behaviour were found to be in agreement with the data from the literature.

Synthesis via the REMA method. – Synthesis of the protected C-terminal hexadecapeptide of secretin, Boc-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, secretin 12–27, was achieved by application of the REMA method. The coupling reactions, the data of which are summarized in Tables 2–4, were carried out according to the following General Procedure.

General procedure. – A 1.4-fold excess of mixed anhydride over amino compound was obtained by adding 1.4 eq of isobutyl chloroformate to a solution of 1.5 eq of N-protected amino acid and 1.5 eq of N-methylmorpholine (NMM) in tetrahydrofuran (THF) or in dimethylformamide (DMF) at a temperature of $-15°$. The time for the formation of the mixed anhydride was 2 min, after which the amino component (1 eq) was added at once. The reaction was performed with mechanical stirring at $-15 \pm 1°$ in a double-walled glass reaction vessel which was kept at the constant temperature by means of a cryostat.

During the coupling 1 µl samples were taken and impregnated on a silicagel plate. After reaction with fluorescamine, the intensity of the spots in long-wave UV. (390 nm) was compared with the intensities obtained from known quantities of amino compound. In this way the amount of amino component still present in the reaction mixture could be estimated. Couplings were continued for 1 h after fluorescamine indicated the presence of less than 0.1% of free amino component.

After a reaction time of 3 h at the utmost the temperature was raised to 0° and a 2M solution of potassium hydrogen carbonate was added until a slightly alkaline reaction was obtained. The mixture was stirred vigorously for 30 min, after which the peptide was precipitated by addition of a 75% saturated aqueous solution of sodium chloride (generally four times the quantity of organic solvent), or in the case of larger peptides by addition of water. The protected peptides, after washing with water, were mostly dried *in vacuo* (at about 0.1 Torr) at room temperature until constant weight. Occasionally preparations still containing moisture were used; this caused no difficulty.

The N-protecting groups were removed by acidolysis, the benzyloxycarbonyl group by dissolving the peptide in 2N hydrogen bromide in acetic acid and keeping the solution at room temperature for 1.5 h. The *t*-butoxycarbonyl group was removed on treatment of the peptide with trifluoroacetic acid at room temperature for 30 min. Deprotected peptides were isolated either by precipitation with ether or by evaporation of the solvent *in vacuo*.

The synthesis described was repeated on a larger scale (about 10 mmol); we obtained 4 grams of secretin 12–27.

L-Amino acid oxidase digestion. – Hydrolysed secretin 12–27 (600 nmol) was dissolved in 0.2 ml of 0.2M 'Trisbuffer' (pH 7.2–7.5). To this mixture a solution of 90 µl of L-amino acid oxidase (*Crotalus Adamanteus*; 4 mg/ml; act. 4.9 units/mg Prot; *Sigma Chemical Company*, St. Louis, U.S.A.) and 5 µl of toluene were added, after which the mixture was shaken in an oxygen atmosphere at 37° for 24 h. The results of the amino acid analysis were compared with those of a hydrolysed mixture of amino acids which was also subjected to the conditions described above.

For the hexadecapeptide digested in this manner no significant racemization within the limits of error ($< 2\%$) for Ala, Val, Leu, and Arg could be detected. Racemization of Ser, Asp, and Glu could not be determined by this method, since they are digested very slowly by L-amino acid oxidase, if at all.

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