($CDCl_3$) δ 1.42 (t), 2.47 and 2.55 (2 s), 4.45 (q), 7.1–8.5 (m), 11.96 (s); MS, *m/e* (relative intensity) 244 (M, 24%), 198 (M – C_2H_5OH, 100).

Acknowledgment. We greatly appreciate the technical assistance of Karen Black, Koni Stone, Mahboob Vora, and Wai-Cheong Wong. This research was supported in part by a grant (AI 13155) from the NIH.

Registry No. **5**, 3123-97-5; **6a**, 88296-04-2; **6b**, 57644-56-1; **6c**, 88296-05-3; **6d**, 88296-06-4; **6e**, 2979-69-3; **7a**, 88296-07-5; **7b**, 88296-08-6; **7c**, 88296-09-7; **7d**, 88296-10-0; **7e**, 88296-11-1; **8a**, 88296-12-2; **8b**, 88296-13-3; **8c**, 88296-14-4; **8d**, 88296-15-5; **8e**, 88296-16-6; **9a**, 88296-17-7; **9b**, 88296-18-8; **9c**, 88296-19-9; **9d**, 88296-20-2; **9e**, 88296-21-3; 11, 16020-16-9; **12b**, 88296-22-4; **12e**, 88296-23-5; 1,3-benzodioxole, 274-09-9; 2,3-dihydrobenzofuran, 496-16-2.

Structure–Activity Studies on the N-Terminal Region of Glucagon

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Using solid-phase methodology and preparative medium- and high-performance reverse-phase liquid chromatography, we have synthesized glucagon and its Arg^{12} analogue in approximately 5% yields. The synthetic glucagon was fully active relative to natural material, and the Arg^{12} peptide exhibited 50% activity. Since perhaps the most critical part of the glucagon-family peptides is the N-terminal hexapeptide region, both batches of resin were split during synthesis in order to prepare two series of analogues based on glucagon and [Arg^{12}]glucagon with changes in the His-Ser-Gln-Gly-Thr-Phe sequence. The following new analogues were tested for their effects on blood glucose levels in normal male rats relative to glucagon and gave the following activities: [Ac-His^1,Arg^{12}]glucagon, 46%; [3-Me-His^1,Arg^{12}]glucagon, 30%; [Phe^1,Arg^{12}]glucagon, 31%; [Des-His^1,Arg^{12}]glucagon, 4%; [D-Ala^2,Arg^{12}]glucagon, 44%; [D-*p*-Cl-Phe^1,D-Ala^4,Arg^{12}]glucagon, 9%; [D-Phe^4]glucagon, 655%; [Ala^2]glucagon, 9%. These data indicate that the amino or imidazole nitrogens of the histidine residue are not essential for biological activity. However, an aromatic group in position 1 may be important, since the Phe^1 analogue is almost as active as glucagon in our bioassay. The superagonist activity with [D-Phe^4]glucagon, which was synthesized to test the hypothesis that a β-bend conformation occurs at this position in glucagon by analogy with luteinizing hormone-releasing hormone and other Gly-containing peptides, indicates that this is indeed the case and has important implications for the receptor-recognition requirements of the glucagon–secretin–vasoactive intestinal peptide family of peptides.

Glucagon, a 29 amino acid hormone (Figure 1), is a member of a closely related group of peptides with a high degree of sequence homology (secretin, VIP,[1] GIP, PHI, and hpGRF) and is well known for its role in the activation of adenylate cyclase,[2] its numerous and well-documented effects upon maintenance of fuel homeostasis, and its possible involvement in the pathogenesis of diabetes mellitus.[3]

A number of studies have been done to evaluate the role of specific amino acids in the interaction of glucagon with its receptor.[4-9] This research was focused on the development of a wide variety of modified derivatives, all of them, with few exceptions,[8] produced by the semisynthetic approach. These were used to investigate the molecular mechanisms involved in glucagon action, as well as to search for clinically useful antagonists of this hormone.

The general conclusion from these numerous studies is that virtually the entire molecule participates in the receptor-recognition process. The entire sequence is also believed to be necessary for the full expression of hormonal activity.[5] However, the lipophilic carboxyl-terminal region appears not to have an essential function in the mechanism of action but is important only for the high receptor affinity of the hormone.[9,10] In fact, the N-terminal hexapeptide of glucagon is all that is required for biological recognition and action.[11] Preservation of the charge of the single lysine at position 12 also appears essential for binding.

Although glucagon, secretin, and VIP have closely related sequences, glucagon is the most effective on hepatic adenylate cyclase and appears to act through a receptor distinct from those that bind secretin and VIP.[12-14] In-

(1) Symbols and abbreviations are in accordance with the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.* **1971**, *247*, 977. Other abbreviations used are VIP, vasoactive intestinal peptide; GIP, gastric inhibitory polypeptide; hpGRF, human pancreatic growth hormone-releasing factor; LH-RH, luteinizing hormone-releasing hormone; HPLC, high-performance liquid chromatography; RP-MPLC, reverse phase medium-pressure liquid chromatography; TFA, trifluoroacetic acid; DMF, dimethylformamide; EDTA, ethylenediaminetetracetic acid.

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Table I. Glycogenolytic Activities of Glucagon Analogues in the Rat

peptide	dose, μg/100 g of body wt	plasma glucose,[a] mg/100 mL	potency,[b] % (95% CL)
saline		133 ± 8	
glucagon	0.5	187 ± 9	
	1	213 ± 8	100
[Ala^2]glucagon (A)	4	176 ± 7	
	8	198 ± 9	9 (7-11)
saline		132 ± 9	
glucagon	0.5	186 ± 10	
	1	214 ± 3	100
[des-His^1,Arg^{12}]glucagon (B)	6	165 ± 7	
	12	186 ± 12	4 (3-6)
[D-*p*-Cl-Phe^1,D-Ala^4,Arg^{12}]glucagon (C)	2	158 ± 8	
	4	176 ± 10	9 (6-14)
saline		124 ± 6	
glucagon	0.5	175 ± 16	
	1	200 ± 10	100
[Phe^1,Arg^{12}]glucagon (D)	1	161 ± 8	
	2	183 ± 10	31 (22-43)
[Arg^{12}]glucagon (E)	0.5	150 ± 12	
	1	176 ± 13	50 (33-76)
[D-Ala^4,Arg^{12}]glucagon (F)	1	174 ± 15	
	2	198 ± 17	44 (32-61)
saline		135 ± 5	
glucagon	0.5	177 ± 13	
	1	199 ± 10	100
[3-Me-His^1,Arg^{12}]glucagon (G)	1	156 ± 3	
	2	183 ± 17	30 (20-43)
[Ac-His^1,Arg^{12}]glucagon (H)	1	170 ± 11	
	2	200 ± 13	46 (35-60)
saline		134 ± 18	
glucagon	0.5	183 ± 8	
	1	215 ± 9	100
[D-Phe^4]glucagon (I)	0.05	171 ± 7	
	0.1	193 ± 13	655 (509-845)

[a] Mean plus or minus SD; $n = 5$. [b] Potency was determined by four-point assay.

H-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-
1 5 10 15

Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-OH
20 25

Figure 1. Amino acid sequence of glucagon.

deed, secretin and VIP do not compete with [^{125}I]glucagon at the glucagon receptor in liver cell membranes. The result of this remarkable specificity is surprising considering the slight variation in sequence at the N-terminal region of these peptides (Figure 2).

With the aim of expanding the investigation into the structural features responsible for the potent glucose-stimulating activity of glucagon, limited until now to only the narrow scope of some semisynthetic analogues, we have synthesized nine analogues of glucagon using solid-phase techniques. The improvements in solid-phase methodology and especially in preparative medium- and high-performance liquid chromatography have made glucagon[15] and other related peptides of this family, such as VIP,[16] secretin,[17] and the recently discovered hpGRF,[18] sufficiently accessible for more intensive analogue work to be undertaken. Since the most critical part of this family of peptides with respect to the transduction of the biological message seems to be the N-terminal hexapeptide region that retains close homology from peptide to peptide, two batches of resin based on glucagon and [Arg^{12}]glucagon were split during synthesis in order to prepare several analogues with changes in the His-Ser-Gln-Gly-Thr-Phe sequence (Figure 1). These new analogues were tested for their effects on glucose levels in normal male rats.

Results and Discussion

Glucagon and its Arg^{12} analogue were synthesized by established methodologies[19] in approximately 5% yields after purification on octadecylsilica. The synthetic glucagon was fully active relative to porcine glucagon (Eli Lilly, Indianapolis), and the Arg^{12} peptide exhibited 50% activity.

The relationships between receptor binding, adenylate cyclase activity, and glycogenolysis in the liver cell are not entirely clear. A number of studies have shown that glucagon analogues that are devoid of adenylate cyclase activity and are potent binding antagonists are still capable of stimulating glycogenolysis.[20,21] Since the aim of glucagon structure–function research is to identify areas of the molecule important in receptor binding and biological activity and thus be able to synthesize superagonists or antagonists of potential therapeutic use, it is obvious that glucagon analogues must eventually be tested for their physiological effects in living animals. In this study we have therefore used a rapid in vivo bioassay as an initial screening procedure to identify analogues of particular interest. In the bioassay used in this study, the activity of synthetic glucagon was indistinguishable from that of

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	1 2 3 4 5 6 7 8
VIP	HIS-SER-ASP-ALA-VAL-PHE-THR-ASP-
SECRETIN	HIS-SER-ASP-GLY-THR-PHE-THR-SER-
PHI	HIS-ALA-ASP-GLY-VAL-PHE-THR-SER-
GIP	TYR-ALA-GLU-GLY-THR-PHE-ILE-SER-
GLUCAGON	HIS-SER-GLN-GLY-THR-PHE-THR-SER-
HPGRF	TYR-ALA-ASP-ALA-ILE-PHE-THR-ASN-

Figure 2. Primary sequence of the first eight residues of the glucagon–secretin family of hormones.

the native hormone (Lilly porcine glucagon). This bioassay, which is a modification of the von Holt et al. method,[22] is simple, rapid, precise, and allows one to screen a large number of glucagon analogues in a short period of time. The fact that other peptides of the glucagon secretin family (PHI, GIP, VIP, hpGRF, and secretin) have only minor effects on blood glucose at pharmacological doses indicates that the assay is specific.

Since several glucagon analogues made using semisynthetic techniques are based on the conversion of lysine to homoarginine in position 12, we synthesized several Arg^{12} analogues with changes in the N-terminal His residue, and the following activities relative to glucagon were found: [$Ac\text{-}His^1$]glucagon, 46%; [3-Me-His^1]glucagon, 30%; [Phe^1]glucagon, 31%; [des-His^1]glucagon, 4%. Our results with [des-His^1,Arg^{12}]glucagon (Table I) are essentially identical with those of Lin et al.[6] with [des-His^1]glucagon prepared by cyanogen bromide cleavage of porcine glucagon. In the latter study, [des-His^1]glucagon was only 2% as active as native glucagon in stimulating adenylate cyclase activity in liver cell membrane.

Substitution of Arg for Lys at position 12 decreases the in vivo biological activity by 50%. [$Homoarginine^{12}$]-glucagon was reported[7] to be a full agonist of glucagon with 20% the potency in the adenylate cyclase assay. Our results with [Arg^{12}]glucagon in vivo also indicate that Lys in position 12 is not essential for biological activity, since maximum stimulation of blood glucose was obtained with this peptide. Apparently, the important point is conservation of the charge at this position. [Des-His^1,$homoarginine^{12}$]glucagon, however, was reported to be only a partial glucagon agonist in that only 22% of maximal adenylate cyclase stimulation could be obtained with the highest dose tested.[7] [Des-His^1,Arg^{12}]glucagon, while only 4% as active as glucagon, stimulated plasma glucose to a level 87% that of the higher dose of glucagon in this assay (Table I), suggesting that this peptide is also a full agonist.

It is clear from these results that the His residue is not at all critical for the activity of glucagon; neither its amino or imidazole nitrogen seems to be essential, since we can derivatize the His residue and still find activity in the peptide. Aromaticity appears to be important, since the Phe^1 analogue, which is a replacement of the histidine residue by another aromatic amino acid, retains almost full agonist activity in our in vivo bioassay. This analogue, however, exhibits a significant reduction in potency (Table I).

Acetylation or methylation of the N-terminal histidine, however, has only a slight effect on potency. These results are in agreement with previous studies in which it has been suggested that while the N-terminal histidine plays an important role in binding and adenylate cyclase activation, it is not essential for biological activity.[6,7]

Of considerable interest in our structure–activity studies on glucagon was the Gly residue in position 4, since it was possible that there could be a β-bend conformation at this position by analogy with LH-RH[23] (in which substitution of Gly for D-Ala results in a considerably more active peptide) and certain other Gly-containing peptides. For example, Gly^4 in secretin has been reported[24] to be replaceable by D-Ala with 10% retention of activity, again suggesting the presence of a β-turn in its closely related sequence. Additionally, something very similar appears to occur with [$D\text{-}Ala^2$]enkephalin.[25]

[$D\text{-}Ala^4$,Arg^{12}]glucagon did indeed exhibit full activity (80%) relative to [Arg^{12}]glucagon, although its potency was significantly reduced. Having in mind our studies on LH-RH (position 6), where replacement of Gly by a bulkier aromatic amino acid produces far greater levels of activity,[26] we made [$D\text{-}Phe^4$]glucagon and found it to be almost 7 times [655% (509–845)] more active than glucagon.

A recent paper described a glucagon analogue, bis[(5-nitro-2-pyrimidyl)ornithine^{17,18}]glucagon, with identical activity with that of glucagon in stimulating adenylate cyclase but only about 50% as potent in the receptor assay. This molecule, however, was more potent than glucagon in stimulating hyperglycemia in the rabbit.[27] The authors suggested that the enhanced activity in vivo could have been due to a decrease in the rate of inactivation of the peptide. Although the duration of action of [$D\text{-}Phe^4$]-glucagon is similar to that of glucagon in our assay (Figure 5), the potency is clearly greater, suggesting that it may be a direct effect on the liver cells rather than the result of decreased enzymatic inactivation. Further studies on the receptor binding in vitro and half-life in vivo of this molecule will be necessary to answer these questions.

In this entire family of peptides, position 4 is occupied by either Gly or Ala. In view of the present result, the presence of absence of the β-bend could contribute enormously to the diverse properties and receptor recognition of these hormones. Replacing serine with alanine in position 2 of glucagon reduces the potency by 90%, but the analogue retains full agonist activity; thus, the hydroxyl group of serine appears to be important for biological activity.

In an approach to the design of antagonist molecules, we synthesized [$D\text{-}p\text{-}Cl\text{-}Phe^1$,$D\text{-}Ala^4$,$Arg^{12}$]glucagon to study the effect of placing a D hydrophobic aromatic amino acid in position 1 instead of the His residue. Substitution of $D\text{-}p\text{-}Cl\text{-}Phe$ for His^2 in LH-RH results in substantial antagonist activity.[28] It can be seen from the biological data (Table I) that our strategy was unsuccessful. Des-(1–4)-glucagon was reported to reduce receptor binding potency to 5.7% that of native glucagon and to abolish both lipolytic and adenylate cyclase activity in vitro. This compound was also shown to be an effective antagonist of glucagon in the adenylate cyclase bioassay.[9] The latter analogue was not, however, tested for glycogenolytic ac-

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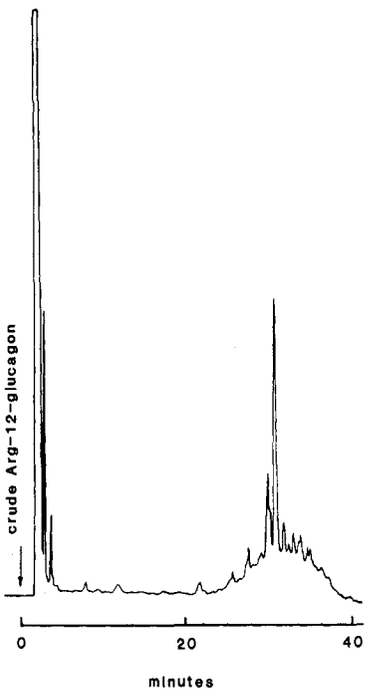

Figure 3. HPLC trace of a sample of crude [Arg[12]]glucagon after Sephadex chromatography on C_{18} Synchropack (6.5 μm, 300 Å; 0.4 × 25 cm) using a linear gradient of 16% to 36% ACN in 0.1% TFA for 30 min: flow rate, 1.5 mL/min; absorption at 215 nm.

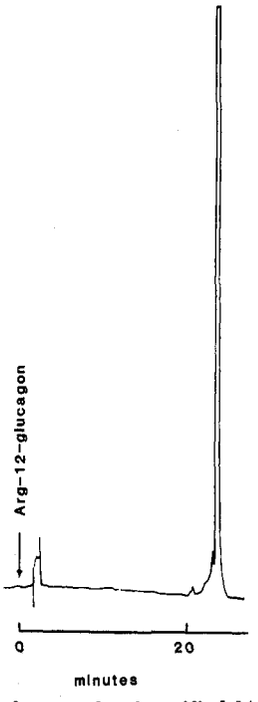

Figure 4. HPLC of a sample of purified [Arg[12]]glucagon from preparative RP-MPLC under conditions described in Figure 3.

tivity. Our results suggest that individual replacement of three of the first four amino acids does not cause a complete loss of biological activity in vivo and that while these four positions may be important in adenylate cyclase activation and receptor binding, they are not essential for glycogenolytic activity.

Experimental Section

Syntheses. Chloromethylated polystyrene resin cross-linked with 1% divinylbenzene was obtained from Pierce Chemical Co., Rockford, IL. Esterification of Boc-Thr(OBzl) to the resin was performed following the Cs salt method.[29] The substitution was 0.8 mequiv/g of resin. All amino acids were coupled as their N^{α}-Boc derivatives (Bachem, Inc, Torrance, CA). Reactive side chains were protected as follows: serine and threonine, benzyl; lysine, 2-chlorobenzyloxycarbonyl; histidine, and arginine, tosyl; aspartic and glutamic acids, *O*-benzyl; tyrosine, 2-bromobenzyloxycarbonyl. The side chains of methionine and tryptophan were left unprotected. The amino acid derivatives were coupled to the Thr(OBzl)-resin in the presence of diisopropylcarbodiimide using a Beckman 990 automatic synthesizer. Boc-Asn and Boc-Gln were coupled in the presence of equimolar amounts of 1-hydroxybenzotriazole; Boc protection was removed at each stage by two treatments with 33% TFA in CH_2Cl_2 for 1 and 25 min. After the addition of Trp,[25] the solvent used for deprotection was changed to 33% TFA, 1% H_2O, and 4% anisole in CH_2Cl_2 until the end of the synthesis. Coupling reactions were monitored at each step by the ninhydrin test[30] and repeated, if incomplete after 60 min, by using the preformed symmetric anhydride procedure[31] in DMF. If free amino groups persisted, they were acetylated with 15% acetic anhydride in CH_2Cl_2 for 20 min.

Cleavage, Deprotection, Purification, and Characterization. The completed, protected peptide-resins, with their N-terminal Boc groups removed to avoid alkylation of methionine during the HF cleavage steps,[32] were deprotected and liberated from the resin support (0.25 mM) by treatment with 15 mL of anhydrous HF containing 10% anisole and 100 mg of dithiothreitol for 1 h at 0 °C. After removal of the HF under nitrogen, the peptides were precipitated with ether, the mixture was filtered, and the peptide was extracted with 50% acetic acid. An HPLC profile of crude [Arg[12]]glucagon is shown in Figure 3. After reduction in volume, the solutions were applied directly onto a 2.5 × 90 cm column of Sephadex G-50 and eluted with 2 N acetic acid. Fractions were collected (200 drops/tube), and aliquots were examined by TLC on Merck silica gel 60 plates in the solvent system 1-butanol–pyridine–acetic acid–water (15:10:3:12). The peptides were visualized by exposure to Ehrlich reagent, pooled, and lyophylized to constant weight. Elution patterns were recorded at 280 nm. These materials were examined by high-performance liquid chromatography (HPLC) on a C_{18} Synchropack (6.5 μm, 300 Å) (0.4 × 25 cm) column using a linear gradient of 16–36% acetonitrile in 0.1% TFA over 30 min with a flow rate of 1.5 mL/min. Absorption at 215 nm revealed a major peak containing contaminants at its leading and trailing edges. The crude products thus obtained were then further purified by reverse-phase medium-pressure liquid chromatography (RP-MPLC) on a 2.5 × 45 cm column of Whatman LRP-1 (C_{18}-bonded silica gel, 13–24 μm) eluted with a linear gradient from 10 to 55% acetonitrile in 0.1% TFA (450 mL each). Aliquots from the main peak were removed and checked by TLC and analytical HPLC under the conditions described above. Those fractions judged pure, favoring purity rather than quantity, were then pooled and lyophylized to constant weight [18–40 mg (2.5–5%) yield based on the concentration (mM) of amino groups substituted onto the starting resin].

With some of the peptides (B, C, F, I), a more rigorous purification was necessary using a C_{18}, 6.5 μm, 300-Å Synchropack column (1 × 25 cm) eluted with a linear gradient of 20–45% acetonitrile in 0.1% TFA developed over 60 min (flow rate 4 mL/min, detection 220 nm). Fractions were collected manually, and aliquots were checked by analytical HPLC. The pure fractions were then pooled and lyophylized to constant weight. The HPLC apparatus consisted of a Altex injector, a Milton Roy pump, and an LKB programmer for a gradient elution. The detection of separated components was carried out with a Holochrome Gilson absorbance detector. All the peptides were detected by their absorbance at 210 nm. Solvents for elution of the peptides were mixtures of acetonitrile (Baker, analyzed reagent) in 0.1% TFA formed in a linear gradient manner from solvent A (0.1% TFA in water) and solvent B (80% acetonitrile in 0.1% TFA). Solvents A and B were filtered immediately prior to use through a 0.5-μm

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Table II. Amino Acid Analyses of Glucagon and Analogues

peptide[a]	Asp	Thr	Ser	Glu	Gly	Ala	Val	Met	Leu	Tyr	Phe	His	Lys	Arg	Trp
Gluc	4.03 (4)	3.00 (3)	3.81 (4)	3.18 (3)	1.00 (1)	1.10 (1)	1.12 (1)	1.12 (1)	2.20 (2)	2.21 (2)	2.11 (2)	0.88 (1)	1.09 (1)	2.11 (2)	0.97 (1)
A	4.12 (4)	2.84 (3)	2.53 (3)	2.99 (3)	1.00 (1)	1.90 (2)	0.84 (1)	0.84 (1)	2.11 (2)	2.03 (2)	1.93 (2)	0.97 (1)	1.08 (1)	2.12 (2)	1.27 (1)
B	4.16 (4)	2.92 (3)	3.47 (4)	3.06 (3)	1.02 (1)	1.00 (1)	0.81 (1)	0.98 (1)	2.06 (2)	2.03 (2)	2.05 (2)			3.05 (3)	1.05 (1)
C	4.11 (4)	2.83 (3)	3.21 (4)	2.93 (3)		1.87 (2)	0.83 (1)	1.00 (1)	2.12 (2)	1.98 (2)	2.03 (2)			3.03 (3)	1.03 (1)
D	3.90 (4)	2.76 (3)	3.28 (4)	2.90 (3)	0.89 (1)	1.00 (1)	0.79 (1)	0.92 (1)	1.94 (2)	1.87 (2)	2.75 (3)			3.03 (3)	1.03 (1)
E	3.89 (4)	2.84 (3)	3.43 (4)	2.88 (3)	0.97 (1)	1.00 (1)	0.97 (1)	0.94 (1)	1.93 (2)	1.94 (2)	1.95 (2)	0.93 (1)		3.04 (3)	0.79 (1)
F	4.10 (4)	3.02 (3)	3.50 (4)	2.92 (3)		1.93 (2)	0.78 (1)	0.97 (1)	2.00 (2)	1.97 (2)	2.00 (2)	0.77 (1)[b]		2.97 (3)	0.18 (1)
G	4.06 (4)	2.97 (3)	3.55 (4)	2.98 (3)	0.97 (1)	1.00 (1)	1.09 (1)	0.92 (1)	2.00 (2)	1.96 (2)	1.95 (2)	0.86 (1)[b]		2.94 (3)	0.83 (1)
H	4.04 (4)	2.94 (3)	3.43 (4)	2.99 (3)	0.95 (1)	1.00 (1)	1.03 (1)	0.91 (1)	2.02 (2)	1.97 (2)	1.97 (2)	0.90 (1)		3.00 (3)	0.79 (1)
I	4.04 (4)	2.82 (3)	3.22 (3)	2.93 (3)		1.00 (1)	0.82 (1)	0.94 (1)	2.03 (2)	1.91 (2)	2.70 (3)	0.81 (1)	0.90 (1)	2.06 (2)	1.17 (1)

[a] Peptides A–I are defined in Table I. D–F were hydrolyzed in 6 M HCl. [b] 3-Me-His.

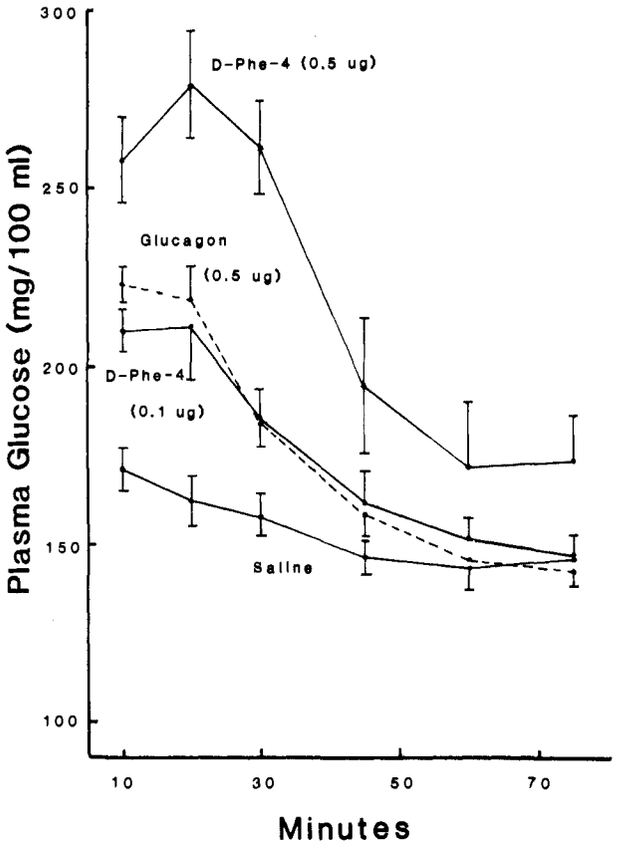

Figure 5. Time-course study on the effect of glucagon and [D-Phe[4]]glucagon on plasma glucose in the anesthetized rat. Points on the graph respresent the mean plus or minus SEM (n = 5). See Experimental Section for details.

Millipore filter and degassed under vacuum. A representative elution profile for purified [Arg[12]]glucagon is shown in Figure 4.

Amino acid analyses were performed on a Beckman Model 119 amino acid analyzer equipped with a System AA computing integrator on samples that were hydrolyzed (110 °C for 18 h) in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole. A modified single-column elution system was used. All amino acid analyses gave expected ratios within ±0.5%, which are shown in Table II. Enzymatic hydrolysis was performed on two samples of glucagon and [Arg[12]]glucagon. The peptides (150 μg) were incubated with amino peptidase M (100%, w/w) for 24 h at 37 °C in 0.2 N N-ethylmorpholine acetate buffer (150 μL), pH 8.1, and the results were as follows: glucagon analyzed as Asp, 3.00 (3); Thr, 3.04 (3); Ser, 3.87 (4); Asn, 1.5 (1); Gln, 2.5 (3); Gly, 1.00 (1); Ala, 1.23 (1); Val, 1.07 (1); Met, 1.00 (1); Leu, 2.03 (2); Tyr, 1.91 (2); Phe, 1.97 (2); Trp, 0.65 (1); His, 0.77 (1); Lys, 1.03 (1); Arg, 2.10 (2). [Arg[12]]glucagon analyzed as Asp, 2.6 (3); Thr, 3.04 (3); Ser, 3.90 (4); Asn, 1.3 (1); Gln, 2.45 (3); Gly, 1.13 (1); Ala, 0.99 (1); Val, 1.00 (1); Met, 1.04 (1); Leu, 2.04 (2); Tyr, 2.03 (2); Phe, 2.01 (2); Trp, 0.87 (1); His, 0.99 (1); Arg, 2.45 (3).

Biology. Male Charles River CD rats weighing 175–350 g were used for all bioassays. The animals were maintained under temperature (24 ± 2 °C) and light (0500–1900 h) conditions for at least 1 week prior to an assay and fed Purina rat chow and tap water ad libitum. All assays were carried out between 1000 and 1200 h. In each assay, groups of five fed rats, anesthetized with sodium pentobarbital (6–7 mg/100 g of body weight), were injected subcutaneously with saline, glucagon, or analogue dissolved in saline 30 min after administration of anesthetic. Peptides were tested at a minimum of two doses. Syringes were coated with a gelatin solution to prevent peptide sticking to the plastic. Injection volume was 0.5 mL. Exactly 15 min after injection of test substance, a 1-mL blood sample was drawn from the jugular vein, transferred to a glass tube containing 1.5 mg of EDTA, and centrifuged. The plasma was drawn off and assayed for glucose by using the glucose oxidase method (Sigma Chemical Co.). Data were analyzed by the four-point assay of Pugsley[33] to determine

potencies. Potencies are expressed as a percentage of glucagon activity in the same assay. Glucagon and [D-Phe⁴]glucagon were also tested for duration of activity (Figure 5) by exactly the same protocol, except that 0.5-mL blood samples were taken at 10, 20, 30, 45, 60, and 75 min after injection of test peptide, 20 min after the sodium pentobarbital dose. One milliliter of saline was injected intraperitoneally after taking the third 0.5-mL blood sample to replace fluid volume.

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Acknowledgment. This research was supported by NIH Grant AM-30167. We thank Ms. Laura Barnes for expert technical assistance.

Registry No. Glucagon, 16941-32-5; [Ala²]glucagon, 88200-39-9; [des-His¹,Arg¹²]glucagon, 88200-40-2; [D-*p*-Cl-Phe¹,D-Ala⁴,Arg¹²]glucagon, 88200-41-3; [Phe¹,Arg¹²]glucagon, 88200-42-4; [Arg¹²]glucagon, 78119-14-9; [D-Ala⁴,Arg¹²]glucagon, 88200-43-5; [3-Me-His¹,Arg¹²]glucagon, 88200-44-6; [Ac-His¹,Arg¹²]glucagon, 88200-45-7; [D-Phe⁴]glucagon, 88200-46-8.

Angiotensin II: Dependence of Hormone Affinity on the Electronegativity of a Single Side Chain[1]

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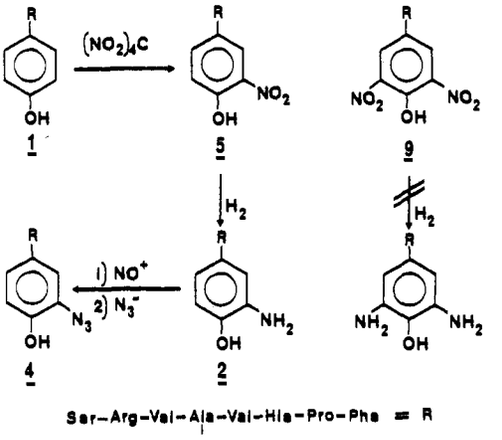
Structure–activity studies on rabbit aorta of angiotensin II analogues have suggested a possible relationship between the electronegativity of the aromatic side chain in position 4 (Tyr) and the observed affinity. In order to test this hypothesis, several other analogues modified in position 4 have been prepared, and all available analogues were tested in three bioassays: in vitro on rabbit aorta strip, in vivo on the rat blood pressure, and the binding assay on beef adrenocortical membranes. In all three bioassays the postulated correlation has confirmed that angiotensin II affinity depends inversely on the electronegativity of the aromatic side chain in position 4.

Angiotensin II [Asp-Arg-Val-Tyr-Val-His-Pro-Phe (AT)] is one of the oldest and best known peptide hormones.[2] Its functions are mainly blood pressure control and are expressed at several sites, to mention only the most important: contraction of blood vessels[3] (increased vascular resistance), stimulation of aldosterone secretion from adrenals[4] (increased sodium uptake), actions on the central nervous system (CNS) to stimulate thirst perception, and centrally mediated blood pressure increase.[5] Many studies have shown the importance of the individual amino acid residues by structure–activity relationships (SAR), but most information was rather crude "yes or no" results. By studying position-4 modified analogues, we found several intriguing points that deserved further investigation:[6] The almost isosteric analogues [Sar¹,Tyr⁴]AT and [Sar¹,(4'-NO_2)Phe⁴]AT differ very much in their biological activity, the first being the "natural" substitution with 100% activity and the second being inactive. Preliminary ^1H and ^{13}C NMR studies[7] have been carried out on these two analogues, but no important conformational difference was visible, and no further data were available. Therefore, it was a still unanswered question if an intra- or intermolecular interaction was the reason for the striking biological difference. We therefore decided to investigate in more detail the possibility of an intermolecular interaction, i.e., the hormone-receptor interaction, involving this particular side chain. The possible interactions were lipophilicity, van der Waals interaction, hydrogen bridge, and electronegativity. The analogues available at this time gave a good correlation for electronegativity, but none was observed for the other parameters. New compounds were added to this correlation[8] and seemed to support this theory, at least on the in vitro bioassay rabbit aorta strip.

Scheme I



This example of electronegativity dependence is the first documented case on peptide hormones according to Pliska[9] and, therefore, had to be investigated in more detail. Two possible benefits of such a study would be (a) superagonists could be found by using amino acid analogues with exceptionally low electronegativity, and (b) if all bioassays indicate similar relationships, we should be able to postulate the identity of AT receptors from different target tissues. In this report we want to present the synthesis of several new, position-4 modified [Sar¹]AT analogues and the biological activity of all available analogues on the three mentioned bioassays and to discuss the results with regard

(1) Abbreviations are according to the IUPAC–IUB Commission for biochemical nomenclature; additional abbreviations are explained in the text. This work has been supported by grants from the Medical Research Council of Canada, the Canadian Heart Foundation, and the Canadian Kidney Foundation. E.E. is a scholar of the Canadian Heart Foundation.

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