Hastings solution as bathing fluid. Milk-ejecting activity was determined on urethane-anesthetized, lactating rabbits following the procedure of van Dyke, *et al.*,[59] as modified by Chan.[60] Avian vasodepressor assays were performed on conscious chickens according to the procedure of Munsick, *et al.*[61] The pressor properties of the polypeptides were determined on atropinized, urethane-anesthetized male rats following the procedures of the U. S. Pharmacopeia.[62] The ability of the polypeptides to enhance water transport (*i.e.*, to induce antidiuresis) was examined in Inactin- and ethanol-anesthetized, hydrated male Sprague-Dawley rats, according to the method of Jeffers, *et al.*,[63] as modified by Sawyer;[64] maximal depression of the rate of urine flow was taken as the effective response. Water transport across the toad urinary bladder was measured according to the method of Bentley[33] as modified by Eggena, *et al.*[34]

Lyophilization, Heat Treatment, Freezing, and Thawing of Asu Analogs. Samples of Asu analogs were prepared for lyophilization experiments in water (pH 6.0) and aqueous triethylamine (pH $\approx$ 9), and for heat treatment in water, as described by Yamanaka, *et al.*[12] After completion of the experiments the stock solutions and experimental samples were diluted identically and assayed for rat pressor activity[62] in the case of Asu-LVP and Asu-AVP and for avian vasodepressor assay[61] in the case of Asu-LVT and Asu-AVT. Another set of aliquots of Asu analogs dissolved in water was frozen in a bath at $-66°$ and thawed three times prior to bioassay; no loss of biological potency was detected. A four-point procedure was used to compare the activities of experimental and control samples. Three low doses of analog (one for each sample) were compared with three high doses at random.

Preparation of Toad Bladder Adenylate Cyclase Extract. Female toads, maintained on moist peat moss until sacrificed, were rapidly pithed and exsanguinated by perfusion through the heart (ventricle) with Ringer's solution (Na^+, 111.1; K^+, 3.5; Cl^-, 116.4; HCO_3^-, 2.4; and Ca^{2+}, 0.89 m*M*; total solute concentration 220 mOsm/kg of H_2O; pH in air 7.8–8.1). Both hemibladders were removed and rinsed with Ringer's solution at room temperature. The epithelial cell layer was scraped off the bladder in a Petri dish maintained on ice with the aid of two glass slides. All subsequent stages of preparation were performed at 0–4°. The cells were collected in 2 ml of 0.225 *M* sucrose containing 0.1 m*M* EGTA and 0.01 *M* Tris-HCl, pH 7.5, and centrifuged at 300 × *g* for 5 min (supernatant discarded). The packed cells were resuspended and again centrifuged as before. They were then homogenized in 2 ml of the same sucrose medium with a tight-fitting glass homogenizer and Teflon pestle and centrifuged at 600 × *g* for 10 min. The supernatant was discarded and the pellet (consisting mostly of broken cell membranes as revealed by a phase contrast microscope) was resuspended in 2 ml of the same medium and centrifuged once more at 600 × *g* for 10 min. The pellet was diluted to a protein concentration[65] of 1–2.5 mg/ml and quick-frozen in an acetone–Dry Ice mixture. The preparation was stored at $-70°$ for up to 4 hr prior to use. A different bladder preparation was used for each independent experiment.

Adenylate Cyclase Assays. Adenylate cyclase activity was assayed according to previously described methods[38,66] with the following modifications: (a) assays were performed at pH 8.0, the optimum pH for neurohypophyseal hormone stimulation;[67,68] (b) incubation periods were 20 min; and (c) the final protein concentration of the toad bladder adenylate cyclase preparation was 0.5–2.0 mg/ml of incubation mixture.

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Secretin. V.[1] Solvent Effects and Conformational Freedom

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Abstract: A comparison of the effects of solvents on the ORD spectra of secretin, glucagon, and lysozyme revealed different types of conformational freedom. In this respect, there is considerable similarity between secretin and several peptides corresponding to longer C-terminal sequences of its chain. The analogy in the solvent-induced conformational change suggests that, in water, the C-terminal part of the hormone is not helical.

Earlier studies of the ORD–CD spectra of the gastrointestinal hormone (porcine) secretin revealed the existence of a preferred conformation in its 27-membered peptide chain. The close similarity of these

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
porcine secretin[2]

spectra with those of (hen egg white) lysozyme indicated the presence of a helical[3] portion in the molecule.

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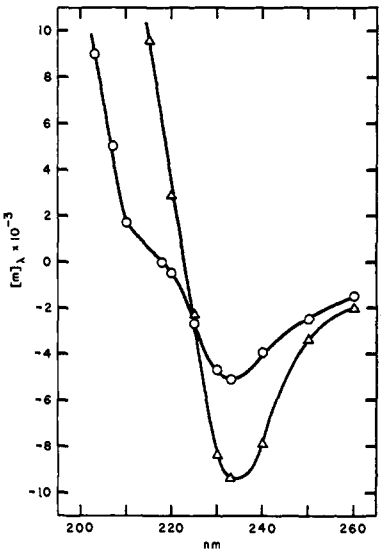

Figure 1. ORD curves of secretin in water, 0.35 mg/ml (○), and in methanol, 0.50 mg/ml (△).

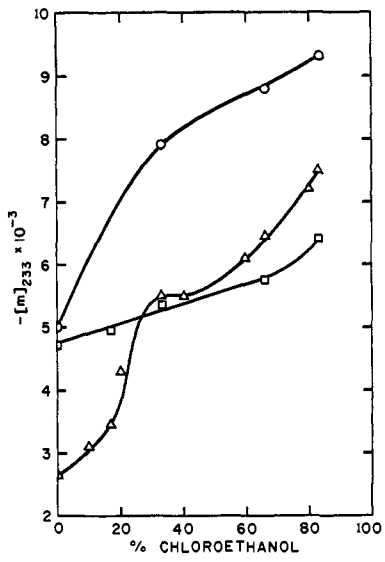

Figure 2. Mean residue rotation at 233 nm of lysozyme, 0.52 mg/ml (□), glucagon, 0.58 mg/ml (△), and secretin, 0.52 mg/ml (○), in water–chloroethanol mixtures.

Examination of the N-terminal and C-terminal halves of the chain, however, failed to determine the position of the helical stretch; in fact, no significant helicicity could be recognized in either half. To reconcile these contradictory findings, a folded model was proposed[1] for secretin, built on the assumption of long-range cooperative interactions between the amino acid residues near each end of the chain. Some experimental support for such a folded, compact molecule was found in the 220-MHz nmr spectrum[4] of secretin and in studies[5] of its diffusion through membranes. Assignment of the helical stretch to the sequence encompassing the amino acid residues 6–13, based on the distribution of nonpolar amino acids[6] and on the analogy with glucagon,[7] remained somewhat speculative and required additional experimentation. This paper reports an attempt to clarify this problem through a study of solvent-induced conformational changes.

The well-known[8] increase in helix content of linear peptides by organic solvents was confirmed in the case of secretin (Figure 1). Preliminary experiments indicated that methanol, trifluoroethanol, and 2-chloroethanol exert similar effects on the ORD spectra. Subsequently, 2-chloroethanol–water mixtures were used, and the changes caused by the organic solvent were followed by plotting the values of mean residue rotation at 233 nm *vs.* chloroethanol concentration. It should be noted, however, that with increasing chloroethanol concentration, not only was the depth of the 233-nm trough increased, but the general character of the spectra was also modified. To establish whether the solvent-induced changes can characterize the conformational freedom of peptide chains, two additional compounds, the pancreatic hormone glucagon and the enzyme (hen egg white) lysozyme, were also examined. Glucagon, already thoroughly studied in this respect by Gratzer and his coworkers,[9] is closely related[7] to secretin and reveals no significant helicicity in its ORD spectra, but can suffer major conformational changes. Lysozyme, a protein that exhibits ORD–CD spectra similar to those of secretin,[1] because it contains β sheets, several short helical areas, and four disulfide bridges,[10] was not expected to undergo gross changes in its architecture on the addition of organic solvents to the aqueous solutions. The comparison of solvent-induced changes in these compounds demonstrated three different types of conformational freedom (Figure 2): rigidity in lysozyme, a great readiness for change at already low chloroethanol concentrations, and also a second rise in helicicity[11] at high organic solvent concentrations in glucagon,[12] and a more limited ability to change its original architecture, in the case of secretin.

The clear-cut differences in the solvent-induced conformational changes in three different molecules (Figure 2) were considered auspicious for an extension of the study to peptides representing partial sequences of secretin.

(3) The expressions "helix," "helical," "helicicity," and "helical stretch" are used throughout this paper to denote a rigid part of a peptide chain that reveals itself in ORD–CD spectra similar in every respect (trough at 233 nm, peak below 200 nm) to those of peptides with well-established helix content. The authors are aware of the need for reservations pointed out by L. C. Craig (*Proc. Nat. Acad. Sci. U. S.*, **61**, 152 (1968)).

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(12) The solvent-induced conformational change of glucagon in Figure 2 is somewhat different from that described by Gratzer and his associates (ref 9). These authors used mixtures of solutions of glucagon in water and in 2-chloroethanol, whereas in the present studies the organic solvent was added to aqueous solutions of the peptides. In some exploratory experiments, solutions of the peptides in chloroethanol were diluted with water and the changes observed were not exactly the same. This hysteresis might be caused by incompletely reversible association of peptide chains in organic solvents.

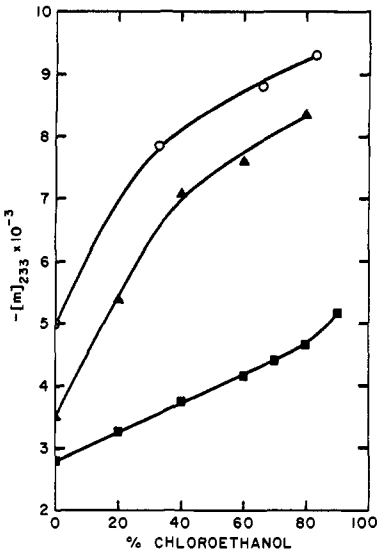

Figure 3. Mean residue rotation at 233 nm of secretin, 0.52 mg/ml (○), S_{14-27}, 0.56 mg/ml (▲), and S_{1-14}, 0.51 mg/ml (□), in water–chloroethanol mixtures.

Experimental Section

Optical rotatory dispersion (ORD) spectra were observed at room temperature with a Carey Model 60 spectropolarimeter, in fused quartz cells of 1- and 5-mm pathlength. Curves were recorded in longer pathlength cells at first. Then when absorption near lower wavelengths became too high, the solutions were transferred to shorter pathlength cells and recording was continued, but starting at higher wavelengths again. Rotation values were calculated from both curves; if values from overlapping portions did not agree, new recordings were made. Base lines were always taken with each run, every cell separate. Because of the low solubility of glucagon (Calbiochem) in water, acetic acid was added to pH 2–3 to prepare a stock solution of 1–2 mg/ml. After passage of the solution through a millipore filter, the concentration was determined from the uv absorption at 278 nm; $E_{1\ cm}^{1\ mg/ml}$ 2.38 was used for calculation.[9] Lysozyme from egg white, recrystallized three times, was purchased from Pentex. Solutions (1–2 mg/ml) were prepared as described for glucagon, and $E_{1\ cm}^{1\ mg/ml}$ 2.63 at 280 nm was used for calculation of the concentration.[13] Aliquots of the stock solutions were diluted with water and with 2-chloroethanol (Matheson Coleman and Bell, Chromatoquality Reagent). Porcine secretin (acetate salt) was a synthetic sample[14] and the peptides representing its partial sequences were obtained from intermediates in the synthesis. Peptides S_{20-27}, S_{17-27}, and S_{16-27} were deprotected by hydrogenation in acetic acid in the presence of a 10% Pd/charcoal catalyst, and were isolated by freeze drying of the concentrated solutions. The peptides were examined by chromatography in a BuOH–pyridine–AcOH–H_2O system[15] on Whatman 3MM paper, and by quantitative amino acid analysis[16] of their hydrolysates on a Beckman Spinco Model 120B analyzer. Concentrations of the aqueous stock solutions of the peptides were calculated from these analyses. In calculating mean residue rotations, $[m]$,[17] no corrections were made for the refractive indices of the solvents.

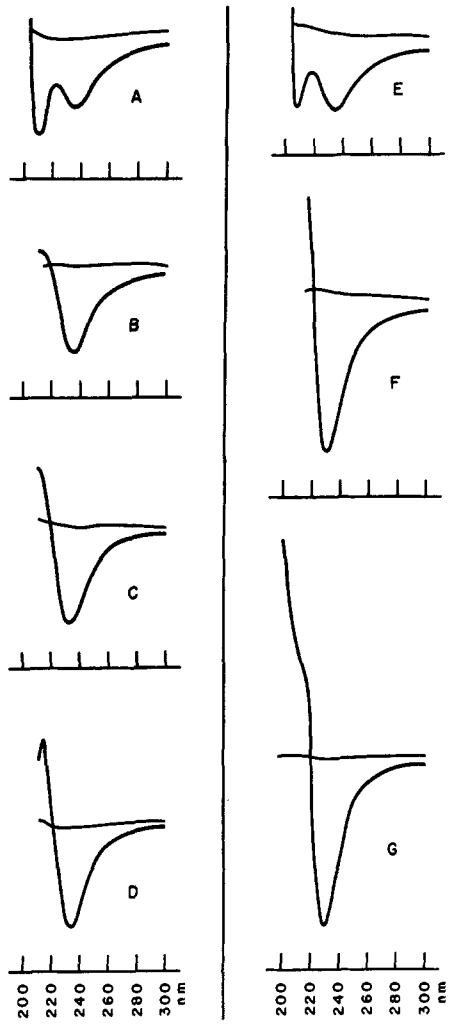

Figure 4. Changes in the character of ORD curves of S_{1-14} (A–D) and S_{14-27} (E–G) on addition of 2-chloroethanol to aqueous solution: A, E, water; B, F, 33% chloroethanol; C, G, 66% chloroethanol; D, 83% chloroethanol.

Results and Discussion

Extensive displacement of water, probably through the decrease of competition for potential intraturn hydrogen bonds, seems to be sufficient for helix formation in many peptides. Indeed, most of the peptides examined in this study gave "helical" ORD spectra in neat chloroethanol or in solvent mixtures with high chloroethanol–water ratios. Therefore, a simple comparison of spectra taken under nonaqueous conditions is not too revealing. More information could be obtained from ORD spectra at lower chloroethanol concentrations. Partial displacement of water by organic solvent seems to produce helices in some chains in which the sequence "cooperates." For the sake of simplicity, a sequence that can be induced to form a helix by moderate concentrations of an organic solvent will be designated as an *S-helicogenic sequence*.[18] The solvent-induced changes in secretin and in two tetradecapeptides, S_{1-14} and S_{14-27}, representing sequences 1–14 and 14–27 of the hormone, are shown in Figure 3, which gives clear evidence of the similarity in respect to

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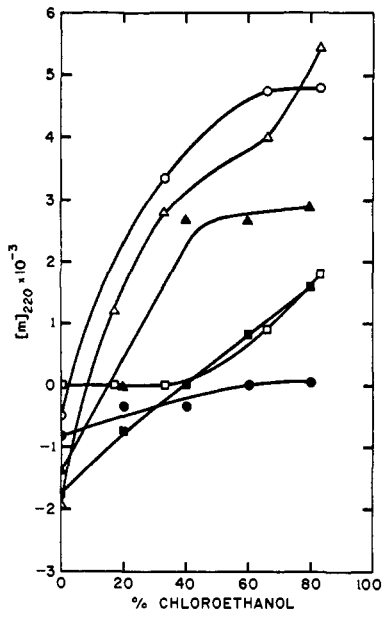

Figure 5. Mean residue rotation at 220 nm of secretin, 0.52 mg/ml (○), glucagon, 0.58 mg/ml (△), lysozyme, 0.52 mg/ml (□), S_{14-27}, 0.56 mg/ml (▲), S_{22-27}, 0.30 mg/ml (●), and S_{1-14}, 0.52 mg/ml (■), in water–chloroethanol mixtures.

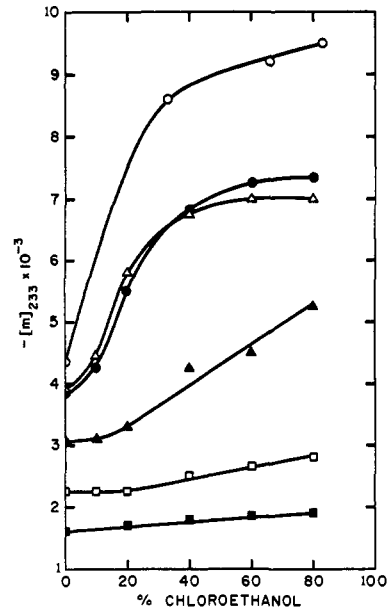

Figure 6. Mean residue rotation at 233 nm of C-terminal peptides of secretin in water–chloroethanol mixtures: S_{5-27} (tricosapeptide), 0.36 mg/ml (○), S_{15-27} (tridecapeptide), 0.40 mg/ml (●), S_{16-27} (dodecapeptide), 0.13 mg/ml (△), S_{17-27} (hendecapeptide), 0.80 mg/ml (▲), S_{18-27} (decapeptide), 0.42 mg/ml (□), S_{22-27} (hexapeptide), 0.30 mg/ml (■).

conformational freedom between secretin and its C-terminal "half" S_{14-27}. Peptide S_{1-14} requires a considerably higher concentration of 2-chloroethanol for a corresponding conformational change, and therefore has no true S-helicogenic sequence. Plotting $[ml_{233}$ *vs.* 2-chloroethanol concentration results in a gross oversimplification, since the solvent-induced changes are not limited to the value of optical rotation at the 233-nm trough. The disappearance of the lower wavelength trough at about 205 nm with the concomitant emergence of a peak below 200 nm (Figure 4) provides more convincing evidence of helix formation. The values of mean residue rotation at 220 nm can be measured with less exactitude than those at 233 nm, but are perhaps more characteristic. The different degrees of conformational freedom in secretin, glucagon, S_{1-14}, S_{14-27}, and S_{22-27} are demonstrated in Figure 5, which has $[m]_{220}$ as the ordinate. A spectrum should not be considered helical if the rotation at 220 nm has a negative value. Of the chains in Figure 5, only glucagon, secretin, and S_{14-27} contain S-helicogenic sequences.

The requirements for S-helicogenic character of S_{14-27} were analyzed by a further anatomy of the secretin chain. The C-terminal hexapeptide S_{22-27}, octapeptide S_{20-27}, decapeptide S_{18-27}, and hendecapeptide S_{19-27} do not show the same readiness to form helices as secretin, the tricosapeptide S_{5-27}, or even the tetradecapeptide S_{14-27} (Figures 3 and 6). Moreover, an S-helicogenic sequence is found in the tridecapeptide S_{15-27} (identical with one of the two thrombinic fragments of secretin[19]). A certain length, perhaps about 12 residues, might be a requirement in S-helicogenic sequences. From the effect of chloroethanol on the conformation of C-terminal sequences of 12 residues or longer, the impression was first gained that the short helical stretch of secretin resides in this part of the molecule, while the N-terminal sequence plays, in water, the stabilizing role. However, the considerable conformational freedom recognized in secretin, and the distinct analogy (*cf.* Figure 3) in the solvent effect between the hormone and its C-terminal sequences, prompted a different interpretation: the solvent-induced conformational change of secretin occurs in the C-terminal half of the chain.

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Conclusion

The N-terminal half of secretin, when separated from the rest of the chain, cannot be induced to form a helix at low concentrations of 2-chloroethanol; its sequence is not S-helicogenic. The C-terminal half of the molecule behaves in this respect very much like the full chain. Therefore, it is reasonable to assume that in aqueous solutions the C-terminal part of the hormone has considerable conformational freedom and, hence, is not helical. Stabilization of the short helix in secretin must rest on more subtle interactions than those provided by the displacement of water with 2-chloroethanol.

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