Optical Rotatory Dispersion and Circular Dichroism of Pepsinogen, Carbamylpepsinogen, and Succinylpepsinogen*  

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SUMMARY

Studies of optical rotatory dispersion (ORD) and circular dichroism (CD) are reported on pepsinogen, carbamyl-, and succinylpepsinogen. From the ORD measurements in the wavelength range of 600 to 300 nm as functions of urea, temperature, and pH, it was shown that the macromolecular conformation of the three proteins differs markedly. On replacing the e-NH₂ groups of the lysine residues of pepsinogen by non-polar groups (carbamylpepsinogen) or acidic side chains (succinylpepsinogen), the electrostatic side chain interaction between the basic residues and some dicarboxylic acids which stabilizes pepsinogen is abolished. With all three proteins the changes in [v], particularly when measured as function of pH, parallel those of the specific rotation, [α]136.

The ORD spectra below 300 μm show several peaks and troughs. The principal trough of the three proteins in phosphate buffer of pH 7.7 and 0.1 ionic strength is at 227 μm and is shifted to 230 μm if the temperature is raised or the pH of the solutions is altered. The positive maximum is at 202 μm for pepsinogen, at 200 μm for carbamylpepsinogen, and at 195 μm for succinylpepsinogen. The ORD spectra above 260 μm show a trough at 278 μm, most likely involving the aromatic side chains. These patterns are very different from those characteristic for α-helical structures.

The CD spectra of the three proteins at pH 7.7 are characterized by a strong negative band at 212, 205, and 202 μm for pepsinogen, the carbamyl, and succinyl derivative, respectively. These are displaced to lower wavelength in the alkaline pH range. The positive CD band of pepsinogen in the phosphate buffer of pH 7.7 is at 193 μm. Above 250 μm, dichroic bands were recorded at 255, 264, 278, 292, and 300 μm. The band at 278 μm is pH-dependent and was assigned to the aromatic amino acid residues. As the pH is raised above pH 10.0, the band passes through zero and changes sign.

ORD spectra have been calculated from the CD data for pepsinogen, carbamyl-, and succinylpepsinogen by the use of a Kronig-Kramers transform with a computer program. Comparison with the observed ORD data shows excellent agreement with the patterns of the observed troughs and peaks. These results are discussed in the light of ORD and CD spectra of known conformations.

In a recent report we have described the optical rotatory dispersion curves of pepsinogen (1, 2). From urea, temperature, and pH studies it was concluded that this protein is stabilized by electrostatic interactions between the basic e-amino groups of the lysine residues, all clustered within the NH2-terminal portion of the molecule, and some of the acidic dicarboxylic acids. To strengthen this argument further, the optical rotatory properties of two modified pepsinogens have been investigated. In these modifications, the e-amino groups of the lysine residues were replaced either by non-polar side chains (carbamylpepsinogen) or by acidic groups (succinylpepsinogen). With these two proteins, the transitions of the specific optical rotation, [α], observed when pepsinogen is taken from the native to the denatured state should have disappeared. In the first part of this communication we will show that this is indeed the case. In the second part of this article we will present the ORD patterns and CD spectra in the wavelength range of 300 to 190 μm which were measured in the presence of urea, at higher temperatures, and in the pH range of 7.0 to 12.0. The optical rotatory dispersion is notably different from that of proteins characterized by high helical content and is reminiscent of that found for the carbonic anhydrases (3, 4), β-lactoglobulins (5), and ribonuclease (6). We also report circular dichroism data for pepsinogen and its derivatives in the native and denatured states. Because the data are complex, our primary aim is to record the results, although only tentative interpretations of the conformation can be offered at this point.

The abbreviations used are: ORD, optical rotatory dispersion; CD, circular dichroism.

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MATERIALS AND METHODS

The pepsinogens used in this research were the Worthington crystalline preparations, lots 119 and 114, with a nitrogen content of 14.82 and 15.01%, respectively, and a molar extinction coefficient of $50.4 \times 10^3$ and $55.0 \times 10^3$ at 278 nm per molecular weight of 40,000. Some of the measurements were made with the chromatographically purified preparations, lots 6LB and 7AA, both having a nitrogen content of 14.80% and a molar extinction coefficient of $51.0 \times 10^3$.

Succinylpepsinogen was prepared by treating pepsinogen (lots 114 and 6LB) with succinic anhydride at pH 8.0 and 15°C, following the procedure described by Gounaris and Perlmann (7).

Carbamylpepsinogen was obtained by reacting pepsinogen (lots 119 and 6LB) with potassium cyanate at pH 8.8 and 30°C (8).

After the reactions had reached completion, the modified proteins were purified by passage over a Sephadex G-25 column, followed by lyophilization of the protein. The number of ε-amino groups of the lysine residues which had reacted with the reagents was determined by the dinitrophenylation procedure of Levy (9) and is given in a later section (Table I).

All chemicals were reagent grade and used without further purification except for urea which was recrystallized twice from 60% ethanol.

Optical Rotatory Dispersion and Circular Dichroism—ORD and CD were measured at 25°C with a Cary model 60 recording spectropolarimeter equipped with the model 6001 CD accessory. Fused quartz cells with 10-mm or 1-mm light paths were used with protein concentrations of 0.5 to 1.0% in the wave length range of 600 to 300 nm and 0.02 to 0.1% below 300 nm. In some experiments, the temperature was varied from 20°C to 70°C. Here, water from an external bath was circulated through jacketed cells to control the temperature to ±0.02°C. All measurements were made after equilibration at each temperature for 15 min, or until an invariant rotation was obtained. In the reversibility experiments, the specific optical rotation of each sample was recorded at the desired temperature. The solutions were then cooled rapidly to 4°C, stored at this temperature for 18 hours, and equilibrated at 25°C prior to the measurement.

The results of the ORD at wave lengths higher than 300 nm are expressed as specific optical rotation, $[\alpha]$, whereas below 300 nm they are given as reduced mean residue rotation, $[m']$, which is related to the specific rotation, $[\alpha]$, by the relation

$$[m']_k = \left( \frac{3}{n^2 + 2} \right) \frac{W_m}{100} [\alpha]$$ (1)

where $[\alpha]$ is the specific rotation at a given wave length, $W_m$ is the mean residue molecular weight taken as 109 (10), and $n$ is the refractive index of the solvent.

The circular dichroism attachment to the Cary model 60 spectropolarimeter records the data directly in terms of ellipticity in degrees, $\theta$. In analogy to $[m']$, $[\theta]$ is degrees cm² per decimole and was corrected for the index of refraction of the solvent.

Theoretical ORD curves were calculated from the CD spectra via the Kronig-Kramers transform which has the form

$$[m'(\lambda)]_k = \frac{2}{\pi} \int_0^\infty [\theta'(\lambda')]_k \frac{\lambda'}{\lambda^2 - \lambda'^2} d\lambda'$$ (2)

Since the indices of refraction of most of the solvents used differ from water, the specific rotation, $[\alpha]$, the mean residue rotation, $[m]$, and the mean residue ellipticity, $[\theta]$, were corrected for the corresponding values in water with the aid of the equation

$$[\alpha]_k^{\text{corrected}} = [\alpha]_k \left( \frac{n_m^2 + 2}{n^2 + 2} \right)$$ (3)

where $n_m$ is the index of refraction of water and $n$ is that of the solvent at $\lambda = 559$ nm. The variation of the index of refraction with wave length was estimated by using the Sellmeier equation (11):

$$n^2 = 1 + \frac{a\lambda^2}{\lambda^2 - \lambda_s^2}$$ (4)

for urea, for instance, $\lambda_s = 115.75$ and $\alpha = 0.9095$.

Viscosity—Specific viscosities were measured at 30°C ± 0.02°C in an Ostwald type viscometer with a flow time for water of approximately 78 sec. Viscosities of each protein solution were obtained at several protein concentrations in the range of 0.5 to 3% and used to compute the intrinsic viscosities.

Protein concentration in the phosphate buffer of 0.032 M Na₂HPO₄-0.004 M NaH₂PO₄, pH 7.7, was determined by the Pregl micro-Kjeldahl method using a mercuric catalyst (12). The nitrogen factor, obtained by independent analyses of nitrogen, ash, and moisture, was used for conversion to dry weight. In the urea-containing solvents or in the glycine buffers, the protein content was estimated from absorbance of the solutions at 278 nm by using the molar extinction coefficient of each pepsinogen preparation for conversion.

The pH of all solutions was measured with the Radiometer pH meter model 4, calibrated with the standard buffers recommended by Bates (13). The pH of the urea-containing solvents represents the apparent pH.

Potential Pepsin Activity—All preparations were assayed for enzymic activity with the hemoglobin method at pH 2.0 (14) after activation of the zymogen at pH 2.0 and 37°C for 10 min.

RESULTS

Optical Rotatory Dispersion of Pepsinogen, Carbamylpepsinogen, and Succinylpepsinogen in Wave Length Range of 600 to 550 nm

The specific optical rotation, $[\alpha]_{366}$, and the dispersion constant, $\lambda_0$, of pepsinogen, carbamylpepsinogen, and succinylpepsinogen, in 0.032 M Na₂HPO₄-0.004 M NaH₂PO₄, pH 7.7, and $\Gamma/2$ 0.1, are given in Table I. This table also includes the number of ε-amino groups of the lysine residues modified, the intrinsic
Table II

Specific optical rotation and rotatory dispersion constant of pepsinogen, carbamyl-, and succinylpepsinogen in urea-phosphate

<table>
<thead>
<tr>
<th>Composition of solvent</th>
<th>Pepsinogen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Carbamyl-pepsinogen</th>
<th>Succinyl-pepsinogen&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha_0$</td>
<td>$\lambda_0$</td>
<td>$\alpha_0$</td>
</tr>
<tr>
<td>No urea beginning</td>
<td>203</td>
<td>236</td>
<td>297</td>
</tr>
<tr>
<td>1.0 M urea-phosphate</td>
<td>203</td>
<td>236</td>
<td>297</td>
</tr>
<tr>
<td>2.0 M urea-phosphate</td>
<td>232</td>
<td>218</td>
<td>316</td>
</tr>
<tr>
<td>4.0 M urea-phosphate</td>
<td>232</td>
<td>218</td>
<td>316</td>
</tr>
<tr>
<td>8.0 M urea-phosphate</td>
<td>232</td>
<td>218</td>
<td>316</td>
</tr>
</tbody>
</table>

<sup>a</sup> Urea, to give the desired concentration, is dissolved in 0.032 M Na$_2$HPO$_4$-0.004 M NaH$_2$PO$_4$ (pH 7.7, 1/2 0.1); the apparent pH values of these solvents vary from pH 7.7 to 7.9.

<sup>b</sup> Taken from Perlmann (1).

<sup>c</sup> The limits of error for $\lambda$ are ±1 mμ but vary about 2 to 3 mμ with the various succinylpepsinogens used in the urea experiments.

viscosity of each protein preparation, and the potential pepsin activity. Several points of interest emerge from these results. The specific rotation, $[\alpha]_{280}$, of carbamyl- and succinylpepsinogen is more levorotatory than that of pepsinogen and the dispersion constant, $\lambda_0$, obtained from the plot of $[\alpha]$, $\lambda_0$ versus $[\alpha]$ (18) decreases from 236 mμ to 220 and 224 mμ for the carbamyl- and succinylpepsinogen, respectively. This low dispersion constant of the modified proteins is close to the $\lambda_0 = 218$ mμ reported for pepsin (16, 17).<sup>2</sup> Furthermore, it is of interest to note that the intrinsic viscosity increases when the e-amino groups of the lysine residues of pepsinogen are either carbamylated or succinylated, clearly indicating that the compactly folded pepsinogen molecules seem to attain a looser and more stretched configuration when the basic e-amino groups of the lysine residues of pepsinogen are either carbamylated or succinylated, clearly indicating that the compactly folded pepsinogen molecules seem to attain a looser and more stretched configuration when the basic e-amino groups are replaced by non-polar or acidic side chains.

Effect of Urea—It has previously been reported that the ORD of pepsinogen changes markedly if the protein is dissolved in concentrated urea solutions with a concurrent sharp transition of $[\alpha]$ in the concentration range of 2.0 to 4.0 M urea. Inspection of Table II reveals that the addition of urea does not alter appreciably the specific rotation, $[\alpha]_{280}$, of carbamyl- and succinylpepsinogen, nor does urea have much effect on $\lambda_0$. Thus the sharp transitions observed with pepsinogen have been abolished.

Effect of Temperature—Since at 25° the optical rotatory properties of carbamyl- and succinylpepsinogen are only slightly affected if these proteins are transferred from an aqueous solvent to urea, the effect of temperature has been investigated as another means of altering the configuration. As shown in Fig. 1, unlike pepsinogen in the sodium phosphate buffer of pH 7.7 where the specific rotation, $[\alpha]_{280}$, decreases markedly in the narrow temperature range of 45–52° with a transition temperature, $T_0 = 47.5$, this temperature-dependent transition has disappeared with the modified proteins. It should be pointed out that, in contrast to the parent protein, $d[\alpha]/dT$ is negative as has also been found with pepsin (17).

Effect of pH—In a previous report it has been shown that altering the pH of a pepsinogen solution affects the optical rotatory properties of this protein in the pH range of 6.8 to 11.2 with a transition midpoint at pH 10.0 (1). Fig. 2 illustrates that with the preparations used in the current research, the change of $[\alpha]_{280}$ occurs in two steps with transition midpoints at pH 9.3 and 10.3, respectively. These results are in accord with those obtained by Herriott (18) and Fratalli, Steiner, and Edelhoch (19) using other criteria. These investigators suggested that the unfolding of pepsinogen at alkaline pH values is a two step...

![Fig. 1. Dependence of specific optical rotation, $[\alpha]_{280}$, of pepsinogen ( ), carbamylpepsinogen (×), and succinylpepsinogen (Δ) on temperature. Measurements were made in 0.032 M Na$_2$HPO$_4$-0.004 M NaH$_2$PO$_4$, pH 7.7, and 1/2 0.1.](image)

![Fig. 2. Dependence of specific optical rotation, $[\alpha]_{280}$, of pepsinogen ( ), carbamylpepsinogen (×), and succinylpepsinogen (Δ) on pH. , results for pepsinogen reported previously (1).](image)
reaction; the one which occurs in the pH range of 8.5 to 10 was found to be reversible. On repeating the ORD measurements on the pepsinogen preparations used previously (lots 6005 and 6012) as function of pH, using the Cary model 60 spectropolarimeter, results identical with those published were obtained (1). This points to the fact that differences exist among some of the pepsinogen preparations which must be due to the handling of the protein during the isolation procedure. Although this explanation of the divergence of the results obtained in our laboratory may not seem wholly satisfactory, in the present discussion it is important to stress the fact that when the positive charges of the e-NH₂ groups of the lysine residues of pepsinogen are abolished either by carbamylation or succinylation, a change of the pH has little effect on [α]₁₃₆⁶ (Fig. 2).

We, therefore, feel that the results described in this section firmly support our original concept that the conformation of pepsinogen is stabilized by side chain interaction of electrostatic nature. On replacing the e-NH₂ groups of the lysyl residues by the non-polar homocitrulline (carbamylpepsinogen) or acidic groups (succinylpepsinogen) the carboxyls of the dicarboxylic acids and also the newly introduced acidic groups of succinylpepsinogen are ionized in the pH range of 7.0 to 12.0. Thus, electrostatic repulsion of the acidic side chains will effect a conformational change toward an "unordered" conformation which is more marked in the case of the succinylated protein. In view of these results it could, therefore, be predicted that the intrinsic viscosity of pepsinogen, carbamylpepsinogen, and succinylpepsinogen should parallel the conformational characteristics of the three proteins. Fig. 3 illustrates that neither carbamyl- nor succinylpepsinogen show a sharp dependence of the intrinsic viscosity on pH. This is in contrast to the observations made with pepsinogen where [η] = 3.0 (g/ml)-¹ in the pH range of 7.7 to 0.2 but subsequently increases sharply to 7.3, 10.9, 12.8, 17.7 (g/ml)-¹ at pH 10.3, 10.7, 11.0, and 11.8, respectively (cf. Reference 19).

The viscosity of succinylpepsinogen at different pH values as a function of concentration is of particular interest. At high pH values and relatively low ionic strength the intrinsic viscosity of succinylpepsinogen has a characteristic polyelectrolyte behavior. The protein molecules thus seem to attain a stretched configuration as a result of electrostatic repulsion between the negatively charged acidic groups, a result reminiscent of that found for poly-L-methionine-S-methylsulphonium bromide (20). ORD and CD of Pepsinogen, Carbamyl-, and Succinylpepsinogen below 300 µm

The ORD curves and CD spectra of pepsinogen, carbamyl-, and succinylpepsinogen in the far ultraviolet may be conveniently divided into two spectral ranges: (a) from 260 to 185 µm and (b) from 300 to 260 µm, where an unusual group of Cotton effects occurs associated with asymmetrical environments of the aromatic side chains and disulfide bonds of proteins (21). a. 260 µm to 185 µm in Phosphate Buffer of pH 7.7 and 0.1 Ionic Strength

Fig. 4A shows the experimental CD spectrum of pepsinogen in phosphate buffer, pH 7.7, and 1/2 0.1, with the characteristic negative trough at 212 µm and a positive Cotton effect at 198 µm. The spectrum crosses the base-line at 200 µm. For the modified proteins the negative band is shifted to 205 and 202 µm for the carbamyl- and succinylpepsinogen, respectively. The corresponding ORD curves are shown in Fig. 4B. With pepsinogen, a negative Cotton effect with a trough at 227 µm was observed which is in good agreement with that obtained by Pollock (22) and also reported by Blout, Pollock, and Parish. The positive maximum is at 202 µm with a cross-over point at 216 µm. The reduced mean residue rotations, [m']₂₃₇ = -3912 to -4169 and [m']₂₃₀ = 7780 ± 300 are in close agreement with those recorded by Pollock (22). Fig. 4B further illustrates that although the negative trough of the Cotton effect of the modified pepsinogens is still at 227 µm, the maxima of the positive peaks are shifted to a lower wave length, i.e. to 200 µm for carbamylpepsinogen and to 195 µm for the succinyl derivative. From Fig. 4 it can also be inferred that the reduced mean residue rotations, [m'], of the three proteins differ only slightly.

In order to verify the proper operation of the instrument, the CD spectra of the three proteins were transformed to the corresponding ORD curves with the aid of the Kronig-Kramers

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**Fig. 3.** Dependence of Intrinsic viscosity, [η], of pepsinogen (●), carbamylpepsinogen (X), and succinylpepsinogen (Δ) on pH.

**Fig. 4.** A, circular dichroic spectra of pepsinogen (●), carbamylpepsinogen (X), and succinylpepsinogen (Δ); B, optical rotatory dispersion calculated by Kronig-Kramers transforms (---) and experimental curves (-----). Measurements were made in 0.092 M Na₂HPO₄, 0.004 M NaH₂PO₄, pH 7.7, and 1/2 0.1.
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FIG. 5. Dependence of optical rotatory dispersion on temperature at pH 7.7. A, pepsinogen; B, carbamylpepsinogen; C, succinylpepsinogen.

TABLE III

Dependence of peaks and troughs in short wave length ORD spectra of pepsinogen, carbamyl-, and succinylpepsinogen on temperature

Solvent: 0.032 M Na2HPO4-0.004 M NaH2PO4, pH 7.7, I/2 0.1.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Temperature</th>
<th>Trough</th>
<th>Peak</th>
<th>Trasform</th>
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<tr>
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<td>227</td>
<td>4199</td>
<td>202</td>
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<tr>
<td></td>
<td>40°</td>
<td>227</td>
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<tr>
<td></td>
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<td>3900</td>
<td>N.R.</td>
</tr>
<tr>
<td></td>
<td>65°</td>
<td>232</td>
<td>3909</td>
<td>N.R.</td>
</tr>
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<td>4240</td>
<td>200</td>
</tr>
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<td></td>
<td>40°</td>
<td>227</td>
<td>4062</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>45°</td>
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<td>195</td>
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<td></td>
<td>55°</td>
<td>232</td>
<td>2908</td>
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<td></td>
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<td>232</td>
<td>2536</td>
<td>N.R.</td>
</tr>
<tr>
<td></td>
<td>65°</td>
<td>232</td>
<td>2940</td>
<td>N.R.</td>
</tr>
<tr>
<td>Succinylpepsinogen</td>
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<td>4000</td>
<td>195</td>
</tr>
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<td></td>
<td>40°</td>
<td>230</td>
<td>4003</td>
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<td>3248</td>
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<td>3026</td>
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<td></td>
<td>65°</td>
<td>232</td>
<td>2890</td>
<td>N.R.</td>
</tr>
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</table>

* N.R., not recorded.

Transform. The calculated ORD patterns are compared with the experimental curves in Fig. 4B. The agreement is excellent. For pepsinogen $[m']_{230}^{25} = -4169$, $[m']_{230}^{35} = -4000$ and $[m']_{230}^{45} = 7780$, $[m']_{250}^{45} = 7200$. A similar good fit was obtained with the carbamyl- and succinylpepsinogen.

Effect of Urea—Urea has a marked effect on the ORD and CD patterns of pepsinogen. In the presence of 8.0 M urea, the Cotton effect at 227 mµ and the CD spectrum at 212 mµ are abolished. Removal of the urea by dialysis of the solution against sodium phosphate buffer of pH 7.7 and 0.1 ionic strength restores the ORD and CD to patterns similar to those recorded in the phosphate buffer (Fig. 4).

Effect of Temperature—The results of measurements in which the temperature of the protein solutions was varied from 30-70° are shown in Fig. 5, A, B, and C. Here, ORD patterns obtained with pepsinogen, carbamylpepsinogen, and succinylpepsinogen at three to four temperatures are superimposed. The noteworthy feature of these patterns immediately becomes apparent. Below 40° the negative trough is at 227 mµ, but it is shifted to 230 mµ when the temperature of the solution is raised. Furthermore, the reduced mean residue rotation, $[m']$, decreases and a broadening of the trough occurs at the higher temperatures. In addition, a shoulder at lower wave lengths appears which is particularly marked with succinylpepsinogen which at 70° has two minima, e.g. at 230 mµ and 208 mµ, respectively, $[m']_{230} = -2500$ and $[m']_{208} = -2000$. The positive peaks are displaced to lower wave lengths. Below 190 mµ the steeply rising absorption at the higher temperatures has hitherto prevented further study. Table III summarizes more detailed data for the positions and magnitudes of the peaks and troughs in the spectra of pepsinogen and the carbamyl and succinyl derivative, respectively. It should be noted that with pepsinogen, $[m']$ changes markedly in the temperature range of 45-50° in which we have observed previously a sharp transition of the specific rotation $[c]_{25}^{15}$ (Fig. 1). On the other hand, the mean residue rotations, $[m']$, of carbamyl- and succinylpepsinogen are altered more gradually. Thus, also the study of the ORD in the far ultraviolet reflects and points to distinct conformational differences of these proteins. Furthermore, the changes produced in the Cotton effects on raising the temperature are reversed upon cooling (1, 2).

Effect of pH—As has already been discussed, in the pH range of 7.0 to 11.0 two pH-dependent conformational transitions occur
in pepsinogen which are reflected in changes of the optical rotational parameters and intrinsic viscosity. Similar, although not as marked, alterations also occur in the ORD patterns of the three proteins in the far ultraviolet. In Fig. 6, A, B, and C, is shown a comparison of ORD patterns of pepsinogen, carbamyl-, and succinylpepsinogen in phosphate buffer of pH 7.7 and in the Sørensen glycine-NaCl-NaOH mixtures at the more alkaline pH values (23). Although not shown in Fig. 6, it was observed that between pH 7.0 and 9.5 no drastic change of the ORD curves occurs with the three proteins. On increasing the pH of the solutions, the troughs at 227 μm broaden and are gradually displaced to 230 μm. A comparison of the reduced mean residue rotations, [m]′, on a wave length basis indicates that the [m]′ values are less negative in the more alkaline pH range. A noteworthy feature of these families of curves is the appearance of a second small Cotton effect below 220 μm. This shoulder becomes more pronounced with the carbamyl and succinyl derivative and occurs at shorter wave lengths, thus, λ = 215 μm and λ = 210 μm for carbamyl- and succinylpepsinogen. Due to the high absorption of the proteins in the NaOH-NaCl-glycine mixtures, the positions of the maxima could not be located at the alkaline pH range. However, that a displacement to shorter wave lengths occurs is manifested by the cross-over points listed in Table IV.

As shown in Fig. 4A the CD spectra of pepsinogen and the modified proteins at pH 7.7 exhibit well marked negative bands at 212, 205, and 202 μm. These bands are gradually displaced toward lower wave lengths in the alkaline pH range which, however, is less noticeable with the modified pepsinogens (Fig. 7). Furthermore, the shoulder that occurs at the high wave length side of the negative Cotton effect, i.e. 217 μm, is more pronounced if the pH of the solution increases.

### Table IV

<table>
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<tr>
<th>Buffer</th>
<th>pH</th>
<th>Cross-over point for</th>
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<tbody>
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<td>216</td>
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<tr>
<td>NaCl-glycine-NaOH</td>
<td>8.3</td>
<td>216</td>
</tr>
<tr>
<td>NaOH</td>
<td>8.7</td>
<td>211</td>
</tr>
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<td>9.8</td>
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<td>10.6</td>
<td>204</td>
<td>207</td>
</tr>
<tr>
<td>11.0</td>
<td>N.C.</td>
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</tr>
</tbody>
</table>

*Composition: 0.032 M Na₂HPO₄, 0.004 M NaH₂PO₄.
*Sørensen's glycine-NaCl-NaOH mixtures, taken from Clark (23).
*N.C., no cross-over.

**b. ORD and CD of Pepsinogen, Carbamylpepsinogen, and Succinylpepsinogen in Wave Length Range of 300 to 260 μm**

The noteworthy feature of the ORD curves and CD spectra of pepsinogen is the occurrence of small Cotton effects in the wave length range of 260 to 320 μm. In the ORD curves in the sodium phosphate buffer of pH 7.7, Γ/2 0.1, a small negative Cotton effect occurs at 278 μm which, however, is hardly noticeable in carbamyl- and succinylpepsinogen. In 8.0 M urea-phosphate, on heating of the protein solution to 50° and in the pH range of 9.6 to 10.0, three of the conditions under which a conformational change of the protein occurs, the negative trough disappears. It can be restored, however, if the solutions are cooled or the pH lowered from 10.0 to 7.7 (cf. References 24 and 25).

It is difficult to specify exactly the location and sign from the ORD measurements alone. These points are, however, more clearly illustrated with the aid of the dichroic spectra the complexity of which is shown in Fig. 8. In addition to the band at 278 μm which most likely is attributable to the aromatic amino acid residues, CD spectra are also recorded at 300, 292, 284, and 255 μm corresponding to the region of absorption of the disulfide bonds (21). These spectra barely appear in solutions of the modified pepsinogens.

The major CD band depicted in Fig. 8 is of particular interest.
FIG. 7. Dependence of circular dichroic spectra on pH. A, pepsinogen; B, carbamylpepsinogen; C, succinylpepsinogen.

FIG. 8. Dependence of circular dichroic spectra of pepsinogen on pH.

FIG. 9. Dependence of ellipticity, \([\theta]_{278}\), of pepsinogen on pH.

in that as the pH of the protein solution is raised above pH 10.0, the band at 278 nm passes through zero and changes sign. Thus, as illustrated with the aid of Fig. 9, the dependence of \([\theta]_{278}\) on pH parallels the pH transition of \([\alpha]_{256}\) (cf. Fig. 2). Lowering the pH retraces the change of \([\theta]\).

DISCUSSION

The study presented in this article has two objectives. Based on the optical rotatory properties of pepsinogen previously reported, the hypothesis had been advanced that the essential elements in the stabilization of this protein, the inactive precursor of the enzyme pepsin, are electrostatic interactions between some of the basic and acidic amino acid residues of the molecule (1).

To strengthen this argument, measurements of the optical rotatory properties of two modified pepsinogens were carried out under identical conditions and were compared with those of pepsinogen. In the modifications chosen the basic e-amino groups of the lysines, all but one clustered within the NH2-terminal portion of the zymogen, were replaced either by non-polar side chains (carbamylpepsinogen) or by acidic groups (succinylpepsinogen). Such a replacement should abolish the electrostatic interactions present in the native protein, thereby changing the configuration and hence, also the optical rotatory properties of the modified pepsinogens.

As shown in the first part of this communication, the sharp transitions of the specific optical rotation, \([\alpha]\), in the wavelength range of 600 to 300 nm, observed when pepsinogen is transferred from an aqueous solvent to urea, on heating of a pepsinogen solution, or on altering the pH, have indeed disappeared with the carbamylpepsinogen and the succinyl derivative. This can be taken as an indication that a conformational change has occurred and that the modified proteins differ considerably from pepsinogen. Furthermore, abolishing some of the positive charges in the native protein by replacing them by non-polar or negatively charged acidic groups must lead to an enhanced electrostatic repulsion of the ionized acidic side chains. Consequently, a

\[ \text{During the succinylation of pepsinogen, 13 to 16 hydroxyamino acid residues are succinylated, thus enhancing the number of negatively charged (acidic) groups in the molecule by 26}\]
indicates structural differences between pepsinogen and its
this region have been described for several proteins (24). In the
range are found with the modified proteins. This further
observed in the CD bands in the far ultraviolet, we feel no suffi-
described for other proteins. Although some fine structure was
bonic anhydrases (3, 4, 28), and the P-lactoglobulins (5, 29).
retained when the e-amino groups of the lysine residues, all
native conformation of pepsinogen. This conformation is not
portion of the molecule which contribute to the Cotton effects
appear not to be affected, at least insofar as alterations in
ORD measurements on the three proteins in the range of 600 to
segment of 41 amino acid residues, the elements within the major
in pepsinogen a negative trough of the ORD patterns ap-
rotatory properties observed are fortuitous and do not reflect the
formations occur in pepsinogen, the helical contributions are at
from "model helix behavior." Hence, if such helical con-
features are associated with the native conformation of pepsinogen. This conformation is not
clustered within 36 amino acids of the NH\textsubscript{2} terminus of the mole-
carried by a drastic increase in the intrinsic viscosi-
Carbamy1- and succinylpepsinogen are characterized by a low
optical rotatory dispersion constant, \( \lambda_{222} \) of 224 and 220 \( \mu \)r, respectively, which is not affected by urea, heat, or change of pH, a behavior similar to that observed with pepsin (16, 17). How-
ever, pepsin has a low intrinsic viscosity, [\( \eta \)] = 3.0 (g/ml)\( ^{-1} \) (20). Therefore, there is no doubt that the similarities of the optical
oratory properties observed are fortuitous and do not reflect the
In the second part of this investigation it has been shown that
the ORD and CD spectra of pepsinogen, carbamylpepsinogen, and succinylpepsinogen in the far ultraviolet are highly charac-
teristic of these particular proteins. The principal features are
that in pepsinogen a negative trough of the ORD patterns ap-
peaks is at 202 nm but is displaced to lower wave lengths when the
protein unfolds. These bands do not correspond to any known
structure and represent a deviation from model helix behavior
similarly, the location of the CD bands also differs from those
for several proteins. In the ORD patterns of the modified pepsino-
gen the negative trough is always at 227 nm, thus resembling ribonuclease (6, 27), the car-
carboxy anhydrases (3, 4, 28), and the \( \beta \)-lactoglobulins (5, 29).
The positive peak, however, is displaced to lower wave length
which is more marked with the succinyl derivative (cf. Table III).
Similarly, the location of the CD bands also differs from those
described for other proteins. Although some fine structure was
observed in the CD bands in the far ultraviolet, we feel no suffi-
cent experimental evidence is available to assign to pepsinogen
definite structural elements such as \( \beta \)-structures, e.g. parallel-
and antiparallel-plated sheet, respectively. One point of
interest, however, emerges from these results. Inasmuch as the
ORD measurements on the three proteins in the range of 600 to
400 nm reflect structural changes induced into a small portion of
molecule, namely into the NH\textsubscript{2}-terminal polypeptide chain
segment of 41 amino acid residues, the elements within the major
portion of the molecule which contribute to the Cotton effects
appear not to be affected, at least insofar as alterations in
second structure large enough to bring about a significant
change in the optical rotatory dispersion are concerned.

In the wave length range of 250 to 300 nm, the presence
of several small bands could be established. The Cotton effects in
this region have been described for several proteins (24). In the
case described here, less intense CD bands in this wave length
range are found with the modified proteins. This further
indicates structural differences between pepsinogen and its
carbamyl and succinyl derivative. Furthermore, disruption of the
"native" structure by heat or alkali leads to disappearance of these Cotton effects as the proteins unfold.

From the foregoing results we can conclude that ORD and CD
curves with characteristic unique features are associated with the
native conformation of pepsinogen. This conformation is not
retained when the \( \epsilon \)-amino groups of the lysine residues, all
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