Several spectral changes have been reported to be related to changes in bonding or other factors in the environment of the side chains of aromatic amino acid residues (1-10). It would then be expected that on addition of a competitive inhibitor to an enzyme solution, a change in the spectrum of the enzyme could be observed, if aromatic amino acid residues were present at the inhibitor-binding site, and interaction took place between these residues and the inhibitor.

During investigation of the relations between the enzymatic activity and the structure of bovine pancreatic carboxypeptidase A, we have found spectral changes due to the interaction between the enzyme and the inhibitor.

Since carboxypeptidase A is scarcely susceptible to the attack of proteolytic enzymes, probably because of the unusually high content of helix, and since chemical modifications of the enzyme do not provide any positive information about the nature of amino acid residues involved in the enzymatic activity, the binding of the inhibitors observed spectrophotometrically was expected to indicate a way to approach the study of the active center of the enzyme. The present paper describes some of our findings.

### Experimental Procedure

**Carboxypeptidase A,** 10 times recrystallized and treated with diisopropyl fluorophosphate, was prepared according to the method of Neurath (11) from frozen bovine pancreas. The protein was homogeneous when examined by ultracentrifugal analysis in 1 mM NaCl-0.05 M Tris buffer at pH 7.5 ($e_{270} = 3.0 S$) and by NH$_2$-terminal analysis.

$\beta$-Phenylpropanoic acid, phenylacetic acid, and benzoic acid were used after recrystallization of the commercial reagents. $n$-Butyric acid was redistilled from the commercial reagent.

The following materials were used as received: $n$-phenylalanine and pepsin, Nutritional Biochemicals Corporation; $\alpha$-tyrosine, Mann Research Laboratories, Inc. (a gift from Ajinomoto Company); $\alpha$-tyrosine and $L$-tryptophan, Ajinomoto Company; hippuric acid, Wako Pure Chemicals Company, Ltd.; lysozyme, Armour and Company. CGP$^*$ was synthesized according to the method of Hofmann and Bergmann (12). All other reagents used were of special grade.

**Enzyme concentrations** were determined spectrophotometrically, assuming the extinction coefficient $E_{157}^1$ to be 19.4 (13). The molecular weight of carboxypeptidase A was assumed to be 34,000 (14, 15).

**Carboxypeptidase activity** was determined by peptidase assay with the substrate CGP in 0.1 M NaCl-0.02 M Veronal buffer of pH 7.5 at 25° (11). Determinations of the inhibitions of the hydrolysis of CGP by $\beta$-phenylpropionate, $\alpha$-phenylalanine, $\alpha$-tyrosine, and $n$-butyrate were carried out according to the procedure of Elkins-Kaufman and Neurath (16).

**Difference Spectra**—Ultraviolet absorption measurements were made with an Ito model QU3 spectrophotometer. Optical density differences between the solutions were measured at room temperature (22-24°) with a set of two well matched 1-cm quartz cells. Into each cell were pipetted 3.00 ml of a sample solution in 1 mM NaCl-0.05 M Tris buffer, pH 7.5, and then 0.01 ml of the inhibitor solution was added to the test solution, and 0.01 ml of water to the reference solution. The difference spectrum was obtained from the optical density difference between the test and reference solutions, by subtraction of the optical density of the inhibitor solution which was measured separately. The difference extinction coefficient ($\Delta e$) was then calculated from the optical density difference ($\Delta D$) as $\Delta D/e$, $e$ being the concentration of carboxypeptidase A in moles per liter.

**Optical rotatory dispersion data** were obtained with a Rudolph model 80S polarimeter at room temperature (21-25°). Measurements were made at the protein concentrations of 0.04% in a cell of 2-cm path length between 250 and 305 m$\mu$, and 0.26% in a 10-cm cell between 305 and 589 m$\mu$.

### Results

**Effects of Competitive Inhibitors on Spectrum of Carboxypeptidase A**—The slight increases in the absorbancy of carboxypeptidase A over the wave length range of 270 to 310 m$\mu$ can be observed when a competitive inhibitor containing a phenyl ring is added to an enzyme solution at pH 7.5. A typical difference spectrum obtained for $n$-phenylalanine is shown in Fig. 1. For all the other inhibitors listed in Table I, identical difference spectra can be obtained in the region 290 to 310 m$\mu$. At the wave length

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* The abbreviation used is: CGP, carbobenzyoxglycyl-$\alpha$-phenylalanine.

**We are indebted to Dr. A. Kato of the Tokyo Industrial Research Institute for making this instrument accessible to us.**
Wave length, mλ

FIG. 1. The extinction coefficient difference (Δε) of carboxypeptidase A (5 × 10⁻⁴ M) on addition of n-phenylalanine (9.9 × 10⁻⁵ M) in 1 M NaCl-0.05 M Tris buffer, pH 7.5, at 22°.

**TABLE I**

**Extinction coefficient difference at 298 mλ (Δε_{0mλ}) of carboxypeptidase A on addition of four competitive inhibitors, and comparison of dissociation constants (K_i) observed spectrophotometrically with those from kinetic studies.**

Difference spectrum measurements were carried out in 1 M NaCl-0.05 M Tris buffer of pH 7.5 at 22-24°, whereas determinations of the inhibitions of the hydrolysis of CGP were in 0.1 M NaCl-0.02 M Veronal buffer of pH 7.5 at 25°.

<table>
<thead>
<tr>
<th>Competitive inhibitor</th>
<th>Range of concentration</th>
<th>Maximal value of Δε_{0mλ}</th>
<th>K_i values</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Phenylpropionate</td>
<td>2-10</td>
<td>6.2 × 10⁻⁴</td>
<td>5 × 10⁻⁴</td>
</tr>
<tr>
<td>Phenyacetate</td>
<td>3-230</td>
<td>3.9 × 10⁻⁴</td>
<td>8 × 10⁻⁴</td>
</tr>
<tr>
<td>α-Phenylalalanine</td>
<td>2-15</td>
<td>2.0 × 10⁻⁴</td>
<td>1 × 10⁻³</td>
</tr>
<tr>
<td>α-Tyrosine</td>
<td>8-50</td>
<td>1.5 × 10⁻³</td>
<td>1 × 10⁻³</td>
</tr>
</tbody>
</table>

* Approximate values.
† From Elkins-Kaufman and Neurath (16), in 0.1 M LiCl-0.04 M phosphate buffer, pH 7.5, at 25°.
‡ Determined at pH 7.0 and 0° by the method of equilibrium dialysis (17).

Above 290 mλ, the slit widths used in obtaining the difference spectra were fairly small (0.04 to 0.4 mm), so experimental errors might be within ±20%. However, in the shorter wave length region, the optical densities of the protein and the inhibitor are very high, so that reliable difference spectra could not be obtained. The maximal Δε is observed at 298 mλ in all cases.

The stoichiometry of the spectral changes is investigated by measuring Δε at 298 mλ as increasing amounts of the inhibitors are added to a solution of carboxypeptidase A of a given concentration. The results for β-phenylpropionate and β-phenylalanine are shown in Fig. 2. The maximal value of Δε at 298 mλ is approximately 200 within experimental error for all the inhibitors listed in Table I.

Beer's law holds at this wave length for β-phenylalanine at the protein concentrations of 4.4 and 6.5 × 10⁻⁴ M as shown in Fig. 2.

**Effect of β-Phenylalanine on Denatured Carboxypeptidase A and Other Proteins—Carboxypeptidase A can be inactivated completely with 3 M guanidinium chloride, or to a considerable degree with Brij 35 (a non-ionic detergent) and by ultraviolet irradiation. Denaturation of carboxypeptidase A abolishes the spectral change found for the native enzyme when n-phenylalanine is added. Neither proteins (pepsin and lysozyme) which are considered not to be inhibited by an inhibitor of carboxypeptidase A, nor a mixture of L-tryptophan and L-tyrosine in the proportion in which they occur in carboxypeptidase A (15), displays any optical density difference at all. The experimental conditions are listed in Table II.**

**TABLE II**

**Experiments on denatured carboxypeptidase A, pepsin, lysozyme, and mixture of L-tryptophan and L-tyrosine**

The buffer and temperature are the same as in Table I.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration of sample</th>
<th>Carboxypeptidase A activity remaining after denaturation</th>
<th>Concentration of p-phenylalanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxypeptidase A treated with Brij-35</td>
<td>3.3 × 10⁻⁴</td>
<td>Almost equal to 0</td>
<td>6.9</td>
</tr>
<tr>
<td>Carboxypeptidase A treated with M guanidinium chloride</td>
<td>6 × 10⁻⁴</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Carboxypeptidase A after ultraviolet irradiation</td>
<td>5.2 × 10⁻⁴</td>
<td>0.2</td>
<td>5-15</td>
</tr>
<tr>
<td>Pepsin*</td>
<td>3.4 × 10⁻⁴</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>Lysozyme†</td>
<td>6.7 × 10⁻⁴</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>A mixture of L-tryptophan and L-tyrosine</td>
<td>1.38 × 10⁻⁴</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>4.31 × 10⁻⁴</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* One mole (36,000 g) of pepsin contains 6 moles of tryptophan and 18 moles of tyrosine (18).
† One mole (14,050 g) of lysozyme contains 5 to 6 moles of tryptophan and 3 moles of tyrosine (19).
‡ Corresponding to the concentrations of L-tryptophan and L-tyrosine, respectively, in carboxypeptidase A of about 2 × 10⁻⁴ M.
Effect of Several Reagents Other than Competitive Inhibitors on Carboxypeptidase A—Experiments carried out in the concentration range of 3 to 4 $10^{-3}$ M of the enzyme and 0.05 to 2 $10^{-3}$ M of each reagent lead to the following conclusion. Benzoate and butyrate, noncompetitive inhibitors of carboxypeptidase A (16), as well as hippurate which does not inhibit the hydrolysis of CGP by the enzyme (16), have no effect on the spectrum of the enzyme at the wave lengths above 290 m$.p$. As indicated by Neurath (20), an effective inhibitor of carboxypeptidase A contains two interacting groups, i.e. an aromatic or heterocyclic ring and a free carboxyl group. Both groups are equally expected to produce such a hyperchromic effect, since environments of high polarizability (2, 3) and carboxylate ions (8) have been found to cause such an effect. To determine whether the carboxyl group is responsible for the observed effect of the competitive inhibitors on the spectrum of carboxypeptidase A, the effect of acetate ion was studied (1.6 $10^{-4}$ M sodium acetate and 3 $10^{-4}$ M carboxypeptidase A). Since the absorbancy of the enzyme does not increase, it seems likely that the difference spectrum (Fig. 1) is attributable to the effect of a phenyl group of the competitive inhibitor on the chromophores of the enzyme molecule.  

Binding of Competitive Inhibitors—On the assumption that only 1 mole of the inhibitor is maximally bound per mole of the enzyme, the relations between the inhibitor concentration and measured $\Delta D$ at 298 m$_{\mu}$ are used to calculate the dissociation constants ($K_i$) for the carboxypeptidase A-inhibitor complexes, i.e. $K_i$ is equal to the concentration of an inhibitor at which 0.5 equivalent is bound.

It can be seen from Table I that $K_i$ values obtained spectrophotometrically agree well with those calculated from the inhibition of the hydrolysis of CGP for $\beta$-phenylpropionate and phenylacetate. In contrast, $K_i$ values for n-phenylalanine and n-tyrosine are not apparently in good agreement with those calculated from the inhibition. It may be that the amino groups of $\alpha$-amino acids produce some extra effect on the chromophores of the enzyme, although we have now no explanation for these discrepancies. In any case, the results may support the view that the interaction between carboxypeptidase A and a competitive inhibitor is responsible for the spectral change.

Ultraviolet Rotatory Dispersion of Mixture of Carboxypeptidase A and $\beta$-Phenylpropionate—The observed spectral changes may be interpreted either as due to the red shift of the absorption bands of the chromophores of the enzyme or as due to the appearance of weak absorption bands. The red shifts of phenolic and indolyl chromophores have been observed in the region of 270 to 295 m$_{\mu}$ when the refractive index or the pH of the solution is increased (1-3, 5, 7-10). The appearance of a new band would be expected to be induced by the specific interaction between the inhibitor and the enzyme accompanying restriction of the free rotation of some of the side chains of the enzyme. For this reason, this new band might be optically active. The ultraviolet rotatory dispersion measurements were carried out to examine whether a Cotton effect can be observed around the absorption band (21). Fig. 3 shows a comparison of the data for carboxypeptidase A in the presence and absence of $\beta$-phenylpropionate, a most powerful and optically inactive inhibitor. The former is different from the latter in that it shows a small Cotton effect near 295 m$_{\mu}$. It then follows that the spectral change at 298 m$_{\mu}$ observed on mixing the enzyme and $\beta$-phenylpropionate may arise from an optically active absorption band near 295 m$_{\mu}$ induced by the binding of the inhibitor.

It may also be noted that the rotatory dispersion of the mixture is essentially identical with that of carboxypeptidase A in the visible and near-ultraviolet regions. Actually, the $b_0$ value is different from the latter in that it shows a small Cotton effect near 295 m$_{\mu}$. It then follows that the spectral change at 298 m$_{\mu}$ observed on mixing the enzyme and $\beta$-phenylpropionate may arise from an optically active absorption band near 295 m$_{\mu}$ induced by the binding of the inhibitor.

There is another possibility that this Cotton effect arises from the absorption band of an aromatic amino acid residue of which the free rotation is restricted by the binding of the inhibitor, since the wave length at which a Cotton effect is observed is not necessarily identical with that of the absorption maximum. A Cotton effect observed at 275 m$_{\mu}$ may be attributed to the oriented phenolic groups on some part of the helical fraction of the enzyme molecule (22).

The data were plotted according to the equation of Moffitt and Yang (20).

$$\frac{3}{n^2 + 2} \frac{M_p}{100} [\alpha] = \frac{a_0 \lambda^2}{n^2 - \lambda^2} + \frac{b_0 \lambda^2}{(\lambda^2 - \lambda_0^2)}$$

in which the first parameter, $a_0$, represents both the intrinsic residue rotations and interactions within the helix, and the second parameter, $b_0$, manifests the excess right-handed helix content; $n$ is the refractive index of the solution. A value of 111 was used for the mean molecular weight per residue, $M_p$, and the arbitrary value of 212 m$_{\mu}$ for $\lambda_0$. 

\( $^6$ The difference spectrum cannot be attributed to the inhibitor, since the substitution of phenyl ring of n-phenylalanine by a phenolic group (n-tyrosine) gave no effect upon the magnitude of the difference spectrum at 298 m$_{\mu}$. \( $^7$ The data were plotted according to the equation of Moffitt and Yang (20).
calculated from the dispersion data (21) is $-260$ in either case. This shows that the over-all configuration of the enzyme has not been modified to a measurable extent by the interaction with $\beta$-phenylpropionate, as previously observed.\footnote{The authors observed that the specific rotation at 589 m\textmu, $\gamma_{\text{D}}$, and the $b_0$ value of a preparation of carboxypeptidase A in sodium chloride solution were $-9^\circ$ and $-430$, respectively, and in 0.06 M $\beta$-phenylpropionate solution at pH 7 it had $[\alpha]_b$ of $-8.7^\circ$ and $b_0$ of $-540$.}

**DISCUSSION**

Powerful competitive inhibitors of carboxypeptidase A, such as $\beta$-phenylpropionate, have a structural feature which resembles the carboxyl-terminal residue (contributing to the susceptible bond) of the typical substrate such as CGP (20). Therefore, it is interesting to study the enzyme-inhibitor interactions spectrophotometrically in the absence of the substrate, although it is not sure that the inhibitor-binding site responsible for the spectral change is identical with the catalytically active site.

Several data obtained here spectrophotometrically indicate the specific interaction between a competitive inhibitor and carboxypeptidase A. (a) The spectral change occurs upon mixing the native enzyme and a competitive inhibitor; (b) the dissociation constant of the enzyme-inhibitor complex determined spectrophotometrically agrees well with those calculated from kinetic studies, although there are some marked exceptions for $\alpha$-amino acids; (c) optical density difference is abolished by denaturation of the enzyme. The discrepancies between the $K_i$ values for the $\alpha$-amino acids, as determined spectrophotometrically and kinetically, are significant, and should be further investigated.

It may also be inferred from the results presented above that the binding must involve a specific aromatic amino acid residue of the enzyme. There is some support for the view that this might be a tryptophyl or tyrosyl residue: (a) maximal spectral differences of the indole chromophores have been observed by several authors at 292 to 295 m\textmu (3, 5, 8, 10), whereas that found here is at 296 m\textmu, which is close to that region; (b) ultraviolet irradiation data\footnote{The authors observed that the specific rotation at 589 m\textmu, $\gamma_{\text{D}}$, and the $b_0$ value of a preparation of carboxypeptidase A in sodium chloride solution were $-9^\circ$ and $-430$, respectively, and in 0.06 M $\beta$-phenylpropionate solution at pH 7 it had $[\alpha]_b$ of $-8.7^\circ$ and $b_0$ of $-540$.} show a possible relation between a tryptophyl or tyrosyl residue and the peptidase activity of carboxypeptidase A. Identification of the binding site is now in progress.

In this respect, it is important to refer to the report of Simmons and Blout (23) which postulates that the protein subunits of the tobacco mosaic virus show the presence of a small inflection point at 283 m\textmu and that this unusual dispersion might be due to a small Cotton effect from oriented aromatic amino acids. A small Cotton effect observed here at 295 m\textmu for the mixture of carboxypeptidase A and $\beta$-phenylpropionate may also be interpreted to arise from the specific orientation of certain aromatic side chains as a result of the strong binding with the aromatic ring of the inhibitor. Possibly, this binding induces a new band corresponding to a charge transfer complex or the $n$-$\pi^*$ transition in an aromatic group. It seems less likely that the Cotton effect at 295 m\textmu is due to the chromophore of $\beta$-phenylpropionate acquiring optical rotatory power on binding to the enzyme, as observed by Styrrer and Blout (24), because the absorption maximum of this inhibitor at pH 7.5 is observed at 260 m\textmu. Unfortunately, we cannot yet discuss these possibilities.

Because of high optical densities of both protein and inhibitor solutions at the wave lengths at which difference spectra of histidines or phenylalanine may be observed (230 to 270 m\textmu) (3, 5, 8), it seems impossible with the present apparatus that they will be observed.

On the other hand, the role of zinc in carboxypeptidase A has been studied by Neurath, Vallee et al. (13), Rupley and Neurath (17), Vallee, Coombs, and Hoch (25) and Coleman and Vallee (26). They have reported that 1 g atom of zinc per mole of the enzyme is responsible for the enzyme activity and it binds the protein through a mercapto group and an $\alpha$-amino group to form an active center of carboxypeptidase A. Furthermore, Rupley and Neurath (17) have found that the metal-free enzyme fails to bind phenylacetate by the method of equilibrium dialysis. The results obtained here have not yet been correlated to the essential zinc atom. The nature of the binding and determination of the binding site in relation to zinc remains to be elucidated. It will be also interesting to apply these methods to carboxypeptidase B (27, 28), because carboxypeptidase A and B show distinct differences in substrate specificity (27, 28), and there is no information about the structural features that are required to determine the substrate specificity. After completion of this manuscript, a report appeared (29) in which the interactions between pancreatic ribonuclease and several nucleotides were observed spectrophotometrically, and it was suggested that the difference spectra at 281 and 286 m\textmu may be due to the changes in the phenolic groups of the enzyme. It seems probable that spectrophotometric investigation of the binding of inhibitors will provide promising data to elucidate the structure of the active center of an enzyme.

**SUMMARY**

Slight increases in the absorbancy of carboxypeptidase A have been found at pH 7.5 at room temperature on addition of four competitive inhibitors: $\beta$-phenylpropionate, phenylacetate, $\alpha$-phenylalanine, and $\alpha$-tyrosine. The spectral changes were greatest at 298 m\textmu in all cases. Within experimental error, an identical extinction coefficient difference at 298 m\textmu (approximately 200) has been observed.

Spectral change was absent when $\alpha$-phenylalanine was added to denatured enzymes or other proteins. Several reagents other than competitive inhibitors also failed to indicate a spectral change.

The calculated dissociation constants of carboxypeptidase A-$\beta$-phenylpropionate and carboxypeptidase A-phenylacetate complexes were in fairly good agreement with those determined from kinetic studies. Ultraviolet rotatory dispersion measurements suggest that the spectral change observed at 298 m\textmu on mixing the enzyme and $\beta$-phenylpropionate may be attributable to an absorption band of the enzyme which exhibits a small Cotton effect near 298 m\textmu.

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Studies on the Binding of Carboxypeptidase A and Several Competitive Inhibitors
Hideko Fujioka and Kazutomo Imahori


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