Probes for Catalytic Action of α-Chymotrypsin in Plastein Synthesis

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A plastein was synthesized with α-chymotrypsin from a dialyzable fraction of a peptic hydrolysate of soybean protein.

The plastein was obtainable also by use of an insoluble preparation of α-chymotrypsin. This may rule out the possibility that the plastein is a product resulting from some chemical peptide-protein (enzyme) aggregation.

No appreciable amount of the plastein was produced when chymotrypsinogen was used instead of α-chymotrypsin.

The plastein synthetic, as well as the protein hydrolytic, activity of α-chymotrypsin was inhibited more or less by a hydrophobic inhibitor (n-hexane), a competitive inhibitor (benzoyl-D,L-phenylalanine), and divalent cations (Zn²⁺, Hg²⁺ and Cu²⁺); the degree of inhibition in each case was approximately similar against both the synthetic and the hydrolytic activities.

Either diisopropylphosphorylation of the β-O of Ser-195 or methylation of the 3-N of His-57 imidazole of α-chymotrypsin repressed the synthetic, as well as the hydrolytic, activity.

Based on these results a possible mechanism was discussed of the plastein synthesis by α-chymotrypsin, especially in relevance to its acylation and deacylation.

α-Chymotrypsin is an authorized “serine” peptidolytic enzyme to which a number of studies have been directed to elucidate its primary structure,⁴° conformation,⁴¹ active site,⁴ and mechanism of catalysis.⁵–⁷ A study by Blow et al.⁷ describes that the peptidolytic process of α-chymotrypsin involves the acyl-enzyme formation at Ser-195 with the aid of the catalytic action by the imidazole of His-57 and the subsequent deacylation resulting from the nucleophilic attack by water activated by the same imidazole. On the other hand, the substrate specificity studies⁸–¹¹ have disclosed the presence of the hydrophobic region (pocket) specific to fitting the aromatic and some aliphatic amino acid residues of substrates. Furthermore, it has been known that several divalent cations affect the electron donating-accepting system of α-chymotrypsin to increase or decrease its hydrolytic activity.¹²

The plastein reaction with α-chymotrypsin has been studied somewhat in earlier days,¹³,¹⁴ but its synthetic process in relation to the necessary conformation, active site, and activation or inhibition features has been remained unclear.

The present study has an aim of solving such unclear points in the α-chymotryptic synthesis of plastein, to elucidate the similarity and dissimilarity, if any, between the synthetic and hydrolytic reactions.

Throughout the paper were used the abbreviations as follows:

Bz-D,L-Phe, benzoyl-D,L-phenylalanine; DIP, diisopropylphosphoryl; DIPF, diisopropyl-
phosphofluoridate; MNBS, methyl p-nitrobenzenesulfonate; TCA, trichloroacetic acid.

MATERIALS AND METHODS

Substrates. A peptic hydrolysate was prepared from the acid-precipitated fraction of soybean protein (denatured) according to Yamashita et al.151 The hydrolysate was dialyzed through a usual cellophane tube against water at 5°C, to obtain a dialyzable fraction which gave only slight turbidity by treatment with TCA. This fraction was freeze-dried and the resulting powdered product was used as the substrate of the plastein synthesis. On the other hand, pig hemoglobin denatured (Difco) was used as the substrate of the α-chymotryptic hydrolysis.

Enzymes. α-Chymotrypsin and chymotrypsinogen (crystalline preparations) were obtained from Sigma Chemical Co. An insoluble (filter paper-bound) α-chymotrypsin was prepared according to the method of Kay and Crook.16 A filter paper (Toyo filter paper No. 2) was alkali-treated and used to bind the enzyme. The protein content of the enzyme paper was 0.095% on a dry-matter basis.

Determination of synthetic and hydrolytic activities. The conditions are outlined as follows. For synthesis: substrate concentration, 50% (w/v); enzyme-substrate ratio, 1/100 (w/w); system pH, 5.0; and temperature, 37°C. For hydrolysis: substrate concentration, 1% (w/v); enzyme-substrate ratio, 1/100 (w/w); system pH, 7.8; and temperature, 37°C. The reaction times were set at 4.5 hr in synthesis and at 30 min in hydrolysis according to the preliminary experiments of obtaining linear time-response relations (Figs. 1 and 2). More detailed descriptions on the procedures, especially on the determination of the respective optical densities (turbidities) and the relative activity, are given as footnotes in each illustration.

Inhibitors. n-Hexane of a reagent grade was used as the hydrophobic inhibitor. Bz-D,L-Phe was used as the competitive inhibitor, which was prepared by coupling benzyol chloride with D,L-phenylalanine by Schotten-Baumann reaction.17, 18

Divalent cations. CaCl₂, ZnSO₄, HgCl₂, and CuSO₄ of a reagent grade were used.

Modifying reagents. DIPF was obtained from a commercial source. MNBS was prepared from p-nitrobenzenesulfonyl chloride and sodium methoxide by the method of Morgan and Cretcher.19

RESULTS AND DISCUSSION

The Plastein reaction is known to be an enzymatic process. This is a major premise on which our present study is undertaken to clarify the reaction mechanism from the aspect of the enzyme function. Accordingly, we should, beforehand, rule out the possibility,

![Fig. 1. Time Course of Plastein Synthesis by α-Chymotrypsin.](image)

An enzyme solution was prepared by dissolving 5 mg of α-chymotrypsin in 0.1 ml of 0.1 M phosphate buffer (pH 7.8) and preincubated at 25°C for 30 min. A substrate solution was prepared by dissolving 500 mg of the substrate (the powdered dialyzable fraction from the peptic hydrolysate of soybean protein) in 0.9 ml of distilled water (final pH 5.0), and preincubated at 37°C for 15 min. Subsequently, a 1:9 mixture of the enzyme and substrate solutions was incubated at 37°C without stirring. Samplings were made at appropriate intervals of the incubation. Each sample was homogenized with 4-fold volume of water using a small blender at 3000 rpm for 1 min. To 0.5 ml of the resulting suspension was added 29.5 ml of aqueous TCA (final concentration 10% in acid), and the mixture homogenized again similarly. The homogeneous suspension was measured for optical density (D) at 600 mμ.
Catalytic Action of $\alpha$-Chymotrypsin in Plastein Synthesis

Fig. 2. Time Course of Protein Hydrolysis by $\alpha$-Chymotrypsin.

An enzyme solution was prepared by dissolving 10 mg of $\alpha$-chymotrypsin in 10 ml of 0.1 M phosphate buffer (pH 7.8) and preincubated at 25°C for 30 min. A substrate solution was prepared by dissolving 1 g of the substrate (denatured hemoglobin) in 90 ml of 0.1 M phosphate buffer (pH 7.8), and preincubated at 37°C for 15 min. Subsequently, a 1:9 mixture of the enzyme and substrate solutions was incubated at 37°C with mild stirring. Samplings were made at appropriate intervals of the incubation. To 0.5 ml of each sample was added 9.5 ml of aqueous TCA (final concentration 3.3% in acid), and the mixture homogenized as described in Fig. 1. The homogeneous suspension was measured for optical density (D) at 600 nm.

If any, that the plastein as precipitated by treatment with TCA may be a product resulting from some chemical peptide-protein (enzyme) aggregation. To make clear this point we used the insoluble enzyme, i.e., the filter-paper-bound $\alpha$-chymotrypsin, because the enzyme is conveniently removable from the system after the reaction. After removing the enzyme from the system and treating the enzyme-free system with TCA, we recognized the occurrence of the precipitation as in the usual case; the relative yield ($V/V_0$) of this precipitate (plastein) was almost comparable with the relative hydrolytic activity of the insoluble $\alpha$-chymotrypsin used (Table I). This result may well enough warrant the usual definition that such a TCA-aided precipitate is a mixture of higher-molecular peptides formed through the enzymatic process.

Table I. Plastein Synthetic and Protein Hydrolytic Activities of an Insoluble Preparation of $\alpha$-Chymotrypsin

For determining the synthetic activity, a piece (105 mg) of the dried enzyme-paper (protein content: 0.095%) was dipped in 1 ml of distilled water containing 500 mg of the substrate (pH 5.0, cf. Fig. 1). The mixture was incubated at 37°C for 4.5 hr and treated as described in Fig. 1 to measure the optical density ($D'$). On the other hand, 0.1 mg of $\alpha$-chymotrypsin dissolved in 1 ml of distilled water was incubated with 500 mg of the substrate (pH 5.0) and treated similarly to obtain the optical density ($D$). The correction factor ($D_0$) was obtained by treating the substrate solution (before incubation) with TCA and by measuring the optical density at the same concentration level (Fig. 1). The relative activity was calculated from $V/V_0 = (D' - D_0)/(D - D_0)$.

For determining the hydrolytic activity, a piece (105 mg) of the dried enzyme-paper was dipped in 50 ml of 0.1 M phosphate buffer (pH 7.8) containing 500 mg of the substrate (cf. Fig. 2). The mixture was incubated at 37°C for 30 min and treated as described in Fig. 2 to measure the optical density ($D'$). On the other hand, 0.1 mg of $\alpha$-chymotrypsin was dissolved in 50 ml of 0.1 M phosphate buffer (pH 7.8) containing 500 mg of the substrate. The mixture was incubated and treated similarly, to obtain the optical density ($D$). The correction factor ($D_0$) was obtained as mentioned above. Thus, the relative activity was calculated from $V/V_0 = (D_0 - D)/(D_0 - D)$.

<table>
<thead>
<tr>
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<th>$V/V_0$</th>
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<tbody>
<tr>
<td>Synthesis</td>
<td>0.158</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td>0.177</td>
</tr>
</tbody>
</table>

As mentioned already, a number of studies have been focussed on $\alpha$-chymotrypsin, elucidating the necessary conformation for this enzyme to exhibit the peptidolytic or hydrolytic activity. In order to probe the effect of the conformational change on the plastein synthetic
activity, we used chymotrypsinogen since this zymogen is unable to shape the active conformation because of lacking in a requirement that an ion pair should be formed between the amino group of Ile-16 and the carboxyl group of Asp-194. As Table II shows, chymotrypsinogen, as expected, was ineffective in producing the plastein; only a slight increase in the relative activity with reaction time may well be neglected. This result suggests that the conformation implicated by the ionic interaction between Ile-16 (an N-terminal) and Asp-194, is required to contribute the plastein synthesis as well as the protein hydrolysis.

A recent paper of Steitz et al. describes that near the catalytic center of α-chymotrypsin there is a hydrophobic pocket into which the hydrophobic side chains of chymotrypsin-specific substrates fit. This pocket, together with the amino acid residues, Ser-214 and Met-192, which are implicated in substrate binding, is considered to play an important role in enhancing the substrate specificity. Another paper disclosed that the hydrolytic activity of α-chymotrypsin showed a more or less degree of decrease by treatment with hydrophobic reagents. On the other hand, Mohammedzadeh-k et al. proposed a simplified method of quantifying the hydrophobic regions of proteins. According to this method we carried out a similar, but somewhat modified, experiment of treating α-chymotrypsin with n-hexane, to probe its synthetic and

**Table II. Plastein Productivity of Chymotrypsinogen**

An enzyme solution was prepared by dissolving 5 mg of chymotrypsinogen in 0.1 ml of 0.1 M phosphate buffer (pH 7.8) and preincubated at 25°C for 30 min. A substrate solution was prepared by dissolving 500 mg of the substrate (cf. Fig. 1) in 0.9 ml of distilled water (final pH 5.0), and preincubated at 37°C for 15 min. Subsequently, a 1:9 mixture of the enzyme and substrate solutions was incubated at 37°C. Samplings were made at 4.5, 8 and 16 hr of the incubation. Each sample was treated as described in Fig. 1 to measure the optical density (D'). A similar experiment was carried out using α-chymotrypsin instead of chymotrypsinogen, to obtain the optical density (D). The correction factor (D₀) was obtained in a similar manner as in Table 1. Thus, the relative activity was calculated from \( V/V₀ = (D' - D₀)/(D - D₀) \).

<table>
<thead>
<tr>
<th>Reaction time, hr</th>
<th>( V/V₀ )</th>
</tr>
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<tbody>
<tr>
<td>4.5</td>
<td>0.000</td>
</tr>
<tr>
<td>8</td>
<td>0.013</td>
</tr>
<tr>
<td>16</td>
<td>0.025</td>
</tr>
</tbody>
</table>

**Table III. Decreases in Plastein Synthetic and Protein Hydrolytic Activities of α-Chymotrypsin by Treatment with n-Hexane**

For determining the synthetic activity, 100 mg of α-chymotrypsin was dissolved in 2 ml of 0.01 M acetate buffer (pH 4.38) in a test tube (1.4 x 10 cm) with a stopper. To the solution was added an excessive amount of n-hexane, and the mixture preincubated for 18 hr at room temperature with mild stirring. Subsequently, the aqueous layer was separated and used as an enzyme solution. A substrate solution was prepared by dissolving 500 mg of the substrate (cf. Fig. 1) in 0.9 ml of distilled water (final pH 5.0), and preincubated at 37°C for 15 min. A 1:9 mixture of the enzyme and substrate solutions was incubated at 37°C for 4.5 hr. The optical densities, \( D, D' \) and \( D₀ \), and the resulting term, \( V/V₀ \), were obtained in a similar manner as in Table I and Fig. 4.

For determining the hydrolytic activity, the above enzyme solution was diluted 50-fold with 0.1 m phosphate buffer (pH 7.8). A substrate solution was prepared by dissolving 1 g of the substrate (cf. Fig. 2) in 90 ml of 0.1 M phosphate buffer (pH 7.8), and preincubated at 37°C for 15 min. A 1:9 mixture of the diluted enzyme solution and the substrate solution was incubated at 37°C for 30 min. The optical densities, \( D, D' \) and \( D₀ \), and the resulting term, \( V/V₀ \), were obtained similarly (Table I and Fig. 4).

<table>
<thead>
<tr>
<th>Activity</th>
<th>( V/V₀ )</th>
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<tbody>
<tr>
<td>Synthesis</td>
<td>0.954</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td>0.964</td>
</tr>
</tbody>
</table>
hydrolytic activity changes caused thereby. After the n-hexane treatment (Table III) the aqueous layer dissolving α-chymotrypsin was directly submitted to gas chromatography and analyzed quantitatively for n-hexane bound with the enzyme. Thus, the quantity was found to be $3.0 \times 10^{-4}$ mg/mg enzyme or ca. 0.08 mole/mole enzyme. Next, we surveyed the ultraviolet difference spectrum to warrant further the occurrence of such binding, and the spectrometry gave the result (Fig. 3) indicating the red-shifting of the absorption by aromatic amino acid residues of α-chymotrypsin due to their exposure to the hydrophobic environment. Concerning the ac-

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**Fig. 3. Difference Spectrum of Hexane-treated α-Chymotrypsin in Reference to Intact α-Chymotrypsin in Acetate Buffer at pH 4.38.**

α-Chymotrypsin (100 mg) was dissolved in 2 ml of 0.01 M acetate buffer (pH 4.38) and treated with n-hexane as described in Table III. The aqueous layer was diluted 50-fold with the same buffer so that the resulting concentration of the enzyme was 0.1% on a weight basis. Using an Hitachi recording spectrophotometer (EPS-3T) the difference spectrum was scanned of this sample in reference to 0.1% intact α-chymotrypsin in the same buffer.

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* A Shimadzu gas chromatograph (GC-1C), equipped with a 3 m column (inside diameter: 3 mm) of silicone SF-96 and with a flame ionization detector, was used, and operated at 30°C with a carrier (N2) at the flow rate of 20 ml/min. Retention time of n-hexane: 7.75 min.
tivity changes in this enzyme by treatment with $n$-hexane we obtained the result (Table III) showing slight, but reproducible effects of repressing both the synthetic and the hydrolytic activities. This result may suggest that, also in the plastein synthesis, the hydrophobic pocket near the catalytic site takes part in specializing and fitting the substrates.

Another inhibitor against $\alpha$-chymotrypsin is Bz-D,L-Phe which has been elucidated by Kaufman et al.\textsuperscript{10)} to inhibit competitively its hydrolytic activity through the formation of the so-called non-productive binding. As Fig. 4 shows, Bz-D,L-Phe has a remarkable influence on $\alpha$-chymotrypsin to decrease its synthetic, as well as hydrolytic activity; the degree of the activity decrease in each case depends linearly on the concentration of this competitive inhibitor (below $5 \times 10^{-2}$ M) until the binding site is fulfilled. This result may be assessed as an evidence ensuring that both the plastein synthetic and the protein hydrolytic processes involve a passing point to which the same catalytic site is relating.

Green et al.\textsuperscript{12)} investigated the effects of divalent cations on the hydrolytic activity of $\alpha$-chymotrypsin against $N$-acetyl-L-tyrosine methyl ester, and clarified their actions as factors influencing either activity increase or decrease. Our present study with their effects on the plastein synthetic and protein hydrolytic activities also gave a similar result (Table IV); $\text{Ca}^{2+}$ contributed to certain degrees of enhancement of the $\alpha$-chymotrypsin activities and others ($\text{Zn}^{2+}$, $\text{Hg}^{2+}$ and $\text{Cu}^{2+}$) to more or less degrees of their inhibition. These divalent cations are considered to cause changes in the conformation of $\alpha$-chymotrypsin in solution and, therefore, to affect favorably or unfavorably its catalytic activity, with formation of some ionic bonding or chelation with the functional groups of the amino acid residues, and such conformational changes are apparently implicated not only in the hydrolysis but also in the synthesis. However, it may be of value to reinvestigate some activity discrepancies observed between the synthesis and the hydrolysis, especially with respect to the effect of $\text{Zn}^{2+}$. One reason considered in this connection is that a part of each cation reacts possibly with substrates, without doing with enzyme, since the substrates (soybean protein hydrolysate) are far lower-molecular than hemoglobin and, hence, bear larger molar fractions of the terminal functional groups.

<table>
<thead>
<tr>
<th>Cation</th>
<th>Concentration (m)</th>
<th>$V/V_0$ Synthetic</th>
<th>$V/V_0$ Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{Ca}^{2+}$</td>
<td>0.01</td>
<td>1.016</td>
<td>1.066</td>
</tr>
<tr>
<td>$\text{Zn}^{2+}$</td>
<td>0.001</td>
<td>0.606</td>
<td>0.196</td>
</tr>
<tr>
<td>$\text{Hg}^{2+}$</td>
<td>0.001</td>
<td>0.000</td>
<td>0.295</td>
</tr>
<tr>
<td>$\text{Cu}^{2+}$</td>
<td>0.001</td>
<td>0.295</td>
<td>0.295</td>
</tr>
</tbody>
</table>
Catalytic Action of \( \alpha \)-Chymotrypsin in Plastein Synthesis

FIG. 5. Inhibition of Plastein Synthetic Activity (○--○) and Protein Hydrolytic Activity (●--●) of \( \alpha \)-Chymotrypsin by Treatment with Diisopropyl-phosphofluoridate.

For determining the synthetic activity, a solution was prepared by dissolving 5 mg of \( \alpha \)-chymotrypsin in 0.09 ml of 0.1 M phosphate buffer (pH 7.8). To the solution was added 0.01 ml of isopropanol containing a given molar concentration of DIPF; the abscissa shows the molar ratio of DIPF ([I]) and \( \alpha \)-chymotrypsin ([E]) where the molecular weight of this enzyme is taken as 25,000. The above mixture was preincubated at 25°C for 20 min and used as an enzyme solution. A substrate solution was prepared and preincubated as described in Fig. 4. Subsequently, a 1:9 mixture of the enzyme and substrate solutions was incubated at 37°C for 4.5 hr. The optical density (D) was measured through the procedures described in Fig. 1. A similar experiment was carried out without using DIPF, to obtain the optical density (D). The correction factor (D) was obtained in a similar manner as in Fig. 4. The relative activity (V/V0) was calculated likewise.

For determining the hydrolytic activity, a solution was prepared by dissolving 10 mg of \( \alpha \)-chymotrypsin in 9 ml of 0.1 M phosphate buffer (pH 7.8). To the solution was added 1 ml of isopropanol containing a given molar concentration of DIPF; the abscissa shows the molar ratio of DIPF ([I]) and \( \alpha \)-chymotrypsin ([E]). The resulting mixture was preincubated at 25°C for 20 min and used as an enzyme solution. A substrate solution was prepared and preincubated as described in Fig. 4. Subsequently, a 1:9 mixture of the enzyme and substrate solutions was incubated at 37°C for 30 min. The optical density (D) was measured as described in Fig. 2. A similar experiment was carried out without using DIPF, to obtain the optical density (D). The correction factor (D) was obtained similarly (Fig. 4). Thus, the relative activity (V/V0) was calculated.

FIG. 6. Inhibition of Plastein Synthetic Activity (○--○) and Protein Hydrolytic Activity (●--●) of \( \alpha \)-Chymotrypsin by Treatment with Methyl-p-nitrobenzenesulfonate.

For determining the synthetic activity, a solution was prepared by dissolving 5 mg of \( \alpha \)-chymotrypsin in 0.091 ml of 0.1 M phosphate buffer (pH 7.8). To the solution was added 0.009 ml of acetonitrile containing a given concentration of MNBS. The resulting mixture was preincubated at 25°C for 1 hr and used as an enzyme solution. A substrate solution was prepared and preincubated as described in Fig. 4. Subsequently, a 1:9 mixture of the enzyme and substrate solutions was incubated at 37°C for 4.5 hr. The abscissa shows the concentration ([I]) of MNBS in this incubation mixture. The optical densities, D, D' and D, and the resulting term, V/V0, were obtained in a similar manner as in Fig. 4.

For determining the hydrolytic activity, a solution was prepared by dissolving 10 mg of \( \alpha \)-chymotrypsin in 9.1 ml of 0.1 M phosphate buffer (pH 7.8). To the solution was added 0.9 ml of acetonitrile containing a given concentration of MNBS. The resulting mixture was preincubated at 25°C for 1 hr and used as an enzyme solution. A substrate solution was prepared and preincubated as described in Fig. 4. Subsequently, a 1:9 mixture of the enzyme and substrate solutions was incubated at 37°C for 30 min. The abscissa shows the concentration ([I]) of MNBS in this incubation mixture. The optical densities, D, D' and D, and the resulting term, V/V0, were obtained similarly (Fig. 4).
Several papers\textsuperscript{3-7} have been presented in relevance to the catalytic site of $\alpha$-chymotrypsin, elucidating that the amino acid residues in this relation are Ser-195 (the target of the acyl-enzyme formation) and His-57 (the general acid-base catalyzer). In addition, Asp-102 is known to participate in the catalytic reaction through the electron-transfer from or to His-57. According to our experiments, the modification of either Ser-195 (with DIPF) or His-57 (with MNBS) of $\alpha$-chymotrypsin led to the effective inhibition of its synthetic, as well as hydrolytic activity (Figs. 5 and 6). In the former case it was observed that the treatment with an equimolar concentration of DIPF resulted in the activity decrease of about 50\%, and its 2-fold concentration had an effect of the almost complete inhibition (Fig. 5). Whilst the formation of the modified enzyme (DIP-chymotrypsin) is likely brought to completion at this point ($[I]/[E]=2$), the latter case (modification with MNBS) shows not so quantitative result and the curves descend asymptotically with increasing concentration of this reagent. The results obtained with these experiments (Figs. 5 and 6) may clearly indicate that, also in the plastein reaction, both Ser-195 and His-57 are of special importance as the factors responsible for the catalytic process.

Information obtained throughout the present study on the plastein synthesis compared with the protein hydrolysis, in relevance to the active site, the hydrophobic region in its neighborhood, the active conformation, and the electron-transfer system, lead us to the tentative conclusion at present that the plastein reaction involves the first step of forming the peptidyl-chymotrypsin at Ser-195 and the subsequent step of its aminolysis or iminolysis (nucleophilic attack) by another peptide with the aid of His-57 serving as a general base catalyzer.

REFERENCES

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18) E. Baumann, \textit{ibid.}, \textbf{19}, 3218 (1886).