On the Reaction of Diazoacetyl Compounds with Pepsin*

ROGER L. LUNDBLAD and WILLIAM H. STEIN

From The Rockefeller University, New York, New York 10021

SUMMARY

Previous studies have shown that diazoacetyl-DL-norleucine methyl ester inactivates pepsin, that Cu(II) greatly facilitates the reaction, and that in the presence of Cu(II) substitution occurs specifically at the carboxyl group of an aspartic acid residue in the enzyme. The present experiments were designed to establish the reasons for the speed and specificity of the reaction and the role of the metal ion. Ag(I) proves to be as effective as Cu(II) in promoting the reaction, whereas Cd(II), Co(II), Pb(II), and Zn(II) are not effective. Au(III) inactivates pepsin in the absence of a diazo compound. If Cu(II) and the diazo compound are mixed prior to the addition of pepsin, the rate of inactivation is increased, suggesting that a reactive complex is formed. The pH optimum for inactivation in the presence of Cu(II) is pH 5.5 to 5.8, whereas with Ag(I) it is pH 4.9. If the diazo compound and Cu(II) are mixed prior to the addition of pepsin, the pH optimum is reduced to about pH 5.0. The diazotized inhibitor need not be a substrate analogue, for diazoacetyl-glycine ethyl ester serves as well as the norleucine derivative. Diazoacetic acid methyl ester also inactivates pepsin rapidly in the presence of Cu(II), but more than 1 eq of reagent is incorporated. Although the rates of inactivation of pepsin prepared from pepsinogen and of commercial pepsin are similar, the stoichiometry of the reaction is not. More diazoacetyl compound is incorporated into commercial pepsin, which is known to be heterogeneous, than into pepsin prepared from pepsinogen.

It is suggested that a copper-complexed carbene is the reactive species. This hypothesis is supported by the finding that dimethylsulfonium phenacylide, which is known to form such a complex, rapidly inactivates pepsin in the presence of Cu(II). Reaction of a carbene is thought to occur at a protonated carboxyl and the specificity is determined by the proximity of an ionized carboxyl which serves to orient the positively charged inhibitor molecule. The proposed mechanism of inactivation is compatible with the presence of at least one active site of pepsin of two carboxyl groups of markedly different pH values. One of these carboxyl groups has a pK higher than either of the two groups which previous kinetic studies have implicated in the activity of the enzyme. It thus appears that there may be at least three carboxyl groups involved in the active site of pepsin.

The action of diphenyldiazomethane on pepsin was studied by Delpierre and Fruton (1). Inactivation of pepsin by diazoacetyl-DL-norleucine methyl ester was described by Rajagopalan, Stein, and Moore (2), who found that in the presence of Cu(II) complete inactivation occurred very rapidly and only 1 eq of norleucine was incorporated per molecule of pepsin. In the absence of Cu(II), inactivation was much slower and incorporation was not stoichiometric. Indirect evidence was presented (2) which suggested that, in the presence of Cu(II), inactivation occurred as a consequence of reaction at a carboxyl group, and Bayliss and Knowles (3) have unequivocally shown that the β-carboxyl group of an aspartic acid residue is involved when pepsin is inactivated by diazoacetyl phenylalanine ester in the presence of Cu(II). Although the nature of the reaction has thus been established, the reasons for its speed and specificity and the role of the metal ion have not. The experiments to be described in this communication were undertaken to shed light on these problems.

While the present studies were in progress, reports from several other laboratories (4–7) have appeared which bear on these questions. For the sake of clarity and brevity these prior results will be discussed at the end of the present communication in conjunction with the data presented here.

EXPERIMENTAL PROCEDURE

Materials—Pepsinogen (Lot PG-6EA) was obtained from Worthington. Sephadex G-25 (bead form, 40 to 210 μ) and sulfoethyl-Sephadex C-25 (bead form, 40 to 120 μ) were products of Pharmacia. Glycylglycine ethyl ester hydrochloride, glycine methyl ester hydrochloride, and DL-norleucine were purchased from the Cyclo Chemical Corporation. Chloroaacetic anhydride was a product of K and K Laboratories, Inc. Dimethyl sulfide (methyl sulfide), 2-bromoacetophenone (phenacyl bromide), and 2,4′-dibromoacetophenone (p-bromophenacyl bromide) were obtained from Matheson, Coleman and Bell. 14C-Labeled diketopiperazine (glycine anhydride-2,5-14C) was obtained from Calbiochem.

* This research was supported in part by a grant from the United States Public Health Service.

† Present address, Dental Research Center, University of North Carolina, Chapel Hill, North Carolina 28002.
and glycine-14C ethyl ester hydrochloride was a product of New England Nuclear. Z-His–Phe–Phe-OMe,1 prepared according to the method of Insouy et al. (8), was a gift of Dr. T. A. A. Dopheide.

Preparation of Pepsin—Pepsin was prepared from pepsinogen by the method of Rajagopalan, Moore, and Stein (9). Worthington's Lot PG-6EA did not activate as had previous batches of pepsinogen. It was difficult to reach the correct pH even when the ratio of HCl to chloroacetic acid was increased as suggested by Rajagopalan et al. Moreover, a heterogeneous product resulted as determined by amino acid analysis and hydroxylapatite chromatography. Ash determinations showed the presence of considerable amounts of inorganic contaminants, probably phosphates. To overcome this difficulty, pepsinogen PG-6EA (500 mg) was desalted by passage at 5°C over a column (2.9 × 37 cm) of Sephadex G-25 previously equilibrated with water. The breakthrough fraction containing the desalted pepsinogen was then activated at 15°C by adjusting to pH 2.0 with chloroacetic acid-HCl (9 ml of 2 N chloroacetic acid + 1 ml of 2 N HCl). After passage over sulfoethyl-Sephadex (cf. Reference 9), it was convenient to desalt a 10-ml aliquot of the pepsin solution by passage at 4°C over a column (2.2 × 37 cm) of Sephadex G-25 previously equilibrated with water. Two-milliliter fractions were collected and the protein concentration was estimated by absorbance at 280 nm with a Zeiss spectrophotometer (ε280 nm = 1.38). The principal effluent peak was collected and the pepsin concentration was adjusted to 2 mg per ml with water. The material was stored in 1.3-ml portions at -20°C until used.

Preparation of Inhibitory Compounds—Diazooacetyl-DL-norleucine methyl ester was prepared by the method of Rajagopalan et al. (2). Glycyl-DL-norleucine was prepared by reacting DL-norleucine with chloroacetic anhydride, followed by amidolysis, as described by Greenstein and Winitz (10). Labeled (two preparations, 12,800 and 10,000 cpm per μmole, respectively) and unlabeled diazoacetylglucine ethyl ester were prepared by diazotizing glycylglycine ethyl ester hydrochloride according to the procedure of Riehm and Scheraga (11). 14C-Glycylglycine ethyl ester hydrochloride was prepared from 14C-diketopiperazine by the method of Riehm and Scheraga (11). 14C-Glycylglycine ethyl ester hydrochloride was prepared from 14C-diketopiperazine by the method of Searle (13).

Dimethylsulfoxonium phenacylidie was prepared from dimethylphenacyl sulfoxonium bromide according to the method of Trost (14). Dimethylphenacyl sulfoxonium bromide was synthesized by the procedure of Böhm and Krause (15).

\[ C_{13}H_{14}O\text{SBr} (261.2) \]

**Calculated**: C 45.97, H 5.02

**Found**: C 46.00, H 5.14

Inhibition Studies—A typical reaction mixture consisted of 0.25 ml of a 0.2% solution (0.015 μmole) of pepsin, 0.05 ml of 0.01 M cupric acetate, 0.10 ml of 0.1 M sodium acetate buffer of the desired pH, and 0.10 ml of methanol containing the compound being tested for inhibitory activity, made to a final volume of 1.0 ml with water. Substitutions in this reaction mixture were made as indicated. The inhibition reactions were performed at 14.5 ± 0.5°C with the diazo compounds or at 37°C with dimethylsulfoxonium phenacylide unless otherwise indicated. At 14°C the temperature was maintained by a cold finger (Forma Scientific Company, Marietta, Ohio, catalogue No. 2523) in conjunction with a circulating water bath (American Instrument Company, model 4-96). At the indicated time intervals, 50-μl aliquots were removed and assayed for enzymatic activity against acid-denatured hemoglobin.

In order to determine the extent of incorporation of 14C-labeled diazo compounds, the experiments were carried out on a larger scale. A solution (3 ml) of desalted pepsin (0.50 μmole), 0.105 ml of 0.1 M cupric acetate (10.5 μmoles), 0.1 ml of methanol containing a 40-fold molar excess unless otherwise indicated (12.18 μmole) of the diazo compound being tested, and 0.3 ml of 0.1 M containing buffer, pH 5.67, were maintained at 14°C for the indicated period of time. Aliquots (30 μl) were assayed for pepsin activity. The reaction mixture was then passed at 4°C over a column (2.9 × 38 cm) of Sephadex G-25 previously equilibrated with 0.05 M NaCl. Two-milliliter fractions were collected and the protein concentration was estimated by absorbance at 280 μg. The protein emerged at the void volume after about 50 min. Radioactivity was determined on a 200- or 250-μl sample withdrawn from the appropriate fractions. (Pepsin activity in these fractions was also checked.) The extent of incorporation was calculated from the radioactivity (4 to 10 times background) in the three fractions containing the highest concentration of pepsin. The results agreed to ±10% and the average figure was used (Tables 1 and 11).

Pepsin Assay and Determination of Radioactivity—Descriptions of these procedures and appropriate references may be found in Rajagopalan et al. (9) and Takahashi, Stein, and Moore (16). The specific activities of the pepsin samples used were the same (±5%) as those given by Rajagopalan et al.

RESULTS

Effect of Metal Ions—Rajagopalan et al. (2) showed that Cu(II) markedly accelerated the rate of the reaction of diazoacetyl-DL-norleucine methyl ester with pepsin. In order to investigate the metal ion specificity of this reaction, a number of other ions have been assayed, including Au(III), Co(II), Pb(II), Cd(II), Zn(II), and Ag(I). The conditions were those given in the legend to Fig. 1 and under “Experimental Procedure.” Pepsin prepared from pepsinogen was used in all cases. Of this group, only silver ions were found to have an effect comparable to that of Cu(II). As can be seen from Fig. 1, Ag(I) at pH 5.0 promotes the inactivation of pepsin by diazoacetyl-DL-norleucine methyl ester somewhat more effectively than does an equimolar amount of Cu(II) at pH 5.6. Auric ions inactivated pepsin irreversibly in the absence of the diazo compounds and the other metal ions at a 66-fold molar excess had no effect whatever in 15 min upon the reaction in the pH range 4.3 to 5.6. Koslov, Ginodman, and Orecikovitch (7), who conducted similar studies, noted that Pb(II) and Ni(II) were ineffective. However, they failed to note the facilitation by Ag(I), doubtless because they carried out the reaction at pH 5.6. As will be reported in a subsequent section, pH has a distinct and somewhat complicated effect upon the reaction.

Effect of Other Diazooacetyl Compounds—In order to study the role of the amino acid moiety, the course of the reaction of diazoacetylglucine ethyl ester and diazoacetic acid ethyl ester were investigated (Fig. 2). The glycine derivative inactivates pepsin prepared from pepsinogen at a rate slightly greater than that observed with the norleucine derivative. The effect of Ag(I) on the reactivity of the glycine derivative is indistinguishable.

---

1 The abbreviations used are: Z, benzoxycarbonyl; Et, ethyl.
The effect of metal ions on the inhibition of pepsin by diazoacetylnorleucine methyl ester. The reactions were carried out with 0.05% pepsin (final concentration 15 μM), pH 5.6 for Cu(II) and pH 5.0 for Ag(I), with a 10-fold molar excess of diazoacetylnorleucine methyl ester (0.15 mM). The molar ratio of metal ion to pepsin in each experiment is given on the figure. The extent of incorporation of 14C-labeled inhibitor is shown in Table I. When pepsin prepared from pepsinogen is inactivated by diazoacetylglycine ethyl ester, approximately 1 eq of reagent is incorporated, just as was previously found to be the case in the reaction of the norleucine derivative (2). If, however, a commercial preparation is used, the extent of incorporation rises to 2 eq. Less incorporation occurs if the commercial material is not purified over Sephadex in order to remove inhibitory peptides. Commercial pepsin has been shown to be heterogeneous, partly as a result of autodigestion. It appears that this heterogeneity results in an increase in the extent of reaction with a diazoacetyl compound.

In the present experiments, the extent of the inhibition of pepsin prepared from pepsinogen is decreased by 90% in the presence of a 33-fold molar excess of the substrate, Z-His-Phe-Phe-OMe (8) (20-fold molar excess of either diazoacetylnorleucine methyl ester or diazoacetylglycine ethyl ester, and a 33-fold molar excess of Cu(II)). However, we find that the enzyme is not protected against these diazo reagents by the competitive inhibitors, iso-amyl alcohol (17) (100- to 1000-fold molar excess) or N-acetyl-L-phenylalanine methyl ester (3) (900-fold molar excess). Hamilton, Spona, and Crowell (8), in their studies with a diazoketone, observed that N-acetyl-L-phenylalaninyl-L-tyrosine protected pepsin from inactivation in the absence of Cu(II) but not when Cu(II) was present. Diazooetric acid methyl and ethyl esters rapidly inactivate pepsin in the presence of cupric ions. Indeed, the rate of inhibition is similar to the corresponding findings with the diazoacetyl amino acid derivatives (cf. Fig. 2). Silver ions failed to promote the reaction of diazoacetic acid ethyl ester with pepsin, however. As can be seen from Table II, the incorporation of ethyl ester is similar to that shown in Fig. 1 for the norleucine derivative. The extent of incorporation of 14C-labeled inhibitor is shown in Table I. When pepsin prepared from pepsinogen is inactivated by diazoacetylglycine ethyl ester, approximately 1 eq of reagent is incorporated, just as was previously found to be the case in the reaction of the norleucine derivative (2). If, however, a commercial preparation is used, the extent of incorporation rises to 2 eq. Less incorporation occurs if the commercial material is not purified over Sephadex in order to remove inhibitory peptides. Commercial pepsin has been shown to be heterogeneous, partly as a result of autodigestion. It appears that this heterogeneity results in an increase in the extent of reaction with a diazoacetyl compound.
tion of reagent, although Cu(II)-dependent, is not as specific as was observed with the diazoacetyl amino acid derivatives. Almost complete inactivation of pepsin prepared from pepsinogen leads to the incorporation of 2 to 3 eq of reagent. With commercial pepsin (purified by passage over Sephadex) the figure rises to 3 to 4 eq. Apparently diazoacetic ester causes some nonspecific esterification.

Effect of Prior Reaction with p-Bromophenacyl Bromide—Erlanger et al. (18) have shown that pepsin can be partially inactivated by p-bromophenacyl bromide. Subsequently, they showed that such partially inactivated pepsin could still react with a diazo compound (19). This finding has been confirmed in the present work. When a p-bromophenacyl bromide-treated sample of commercial pepsin was allowed to react with diazoacetylglycine ethyl ester at 15° under the standard conditions (Table I), the remaining activity (20 to 25%) was lost in 15 min. Cupric ions were essential. When the extent of incorporation of the reagent was investigated, it was found to be about 1 residue per molecule, which is less than the 2 residues per molecule observed with untreated commercial pepsin (Table I).

Interaction of Metal Ions with Diazoacetyl Compounds—If alcoholic solutions of cupric ions and of diazoacetyl glycine ethyl ester are mixed and allowed to stand at 14° for 15 min prior to the addition of pepsin, inactivation is so greatly accelerated that after 2 min only one-third of the initial enzyme activity remains (Fig. 3). Clearly, a highly reactive intermediate is formed by the interaction of Cu(II) and the diazoacetyl derivative. Kozlov et al. (7) reached a similar conclusion. They suggested, as had Delpierre and Fruton (4), that Cu(I) and metallic copper may play a role in the reaction. We have no information on this point.

The present findings are different from those of Rajagopalan et al. (2) who reported that prior admixture of cupric ions and pepsin could eliminate the lag phase observed in the loss of activity upon the addition of diazoacetyl-DL-norleucine methyl ester. We have not been able to reproduce this effect.

The formation of an active complex of Cu(II) and diazoacetyl amino acids is supported by studies on the effect of increasing the Cu(II) concentration upon the rate of the inactivation of pepsin by a 10-fold molar excess of diazoacetyl-DL-norleucine methyl ester. If the molar excess of Cu(II) is progressively increased from 16.5 to 33, 66, 132, and 198, the rate of inactivation rises until a 132-fold excess is reached and then levels off. This rise is probably a reflection of an increase in the rate of formation of the reactive intermediate.

Effect of pH upon the Reaction—The extent of inactivation of pepsin by diazoacetyl-norleucine methyl ester as a function of pH is shown in Fig. 4. The pH-rate profiles were determined in acetate and formate buffers. Nonprotein carboxyl groups do not compete for the reagent, however, for the rate is the same if chloride is substituted for acetate. The rate of the reaction is decreased by increasing the ionic strength whether by chloride or by acetate.

![Fig. 3. The effect of prior mixing of cupric ions and diazoacetylglycine ethyl ester upon the rate of pepsin inhibition. The mixture consisted of 2.0 mg of diazoacetylglycine ethyl ester in 0.7 ml of methanol and 0.3 ml of 0.01 M ethanolic cupric acetate. At zero time (O---O) and after 15 min (●—●) at 14°, 0.1 ml aliquots were added to a pepsin solution containing 0.25 mg of pepsin (2.0 mg per ml), 0.1 ml of 0.12 M acetate buffer, pH 5.42, and 0.55 ml of water.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Source of pepsin</th>
<th>Metal ion</th>
<th>Inhibitor incorporated</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepsinogen</td>
<td>None</td>
<td>0.0</td>
<td>0%</td>
</tr>
<tr>
<td>Pepsinogen</td>
<td>Cu(II)</td>
<td>2.8</td>
<td>85%</td>
</tr>
<tr>
<td>Pepsinogen</td>
<td>Cu(II)α</td>
<td>2.3</td>
<td>85%</td>
</tr>
<tr>
<td>Pepsinogen</td>
<td>Ag(I)</td>
<td>0.0</td>
<td>0%</td>
</tr>
<tr>
<td>Commercial (over Sephadex)</td>
<td>Cu(II)</td>
<td>2.9</td>
<td>85%</td>
</tr>
<tr>
<td>Commercial (over Sephadex)</td>
<td>Cu(II)α</td>
<td>4.3</td>
<td>0%</td>
</tr>
<tr>
<td>Commercial (not over Sephadex)</td>
<td>Cu(II)</td>
<td>1.4</td>
<td>0%</td>
</tr>
</tbody>
</table>

a Reaction time, 2 hours.
b pH = 5.00.
c Reaction temperature, 37°.
d Not determined.
Fig. 4. The effect of pH on the inhibition of pepsin by diazo-acetylnorleucine methyl ester in the presence of Cu(II) and Ag(I). The reactions were performed as described in the legend to Fig. 1 except for the use of acetate or formate buffers (constant ionic strength) of the indicated pH. Cu(II) and Ag(I) were present in a 66-fold and 33-fold molar excess (1 mM and 0.5 mM), respectively. Each point is an average of either two or four individual determinations of activity. The relative extent of inactivation is the inhibition after 2 min of reaction divided by the inhibition observed in 2 min at pH 4.8 for Ag(I) and pH 5.6 for Cu(II).

Fig. 5. The effect of prior mixing of Cu(II) and diazoacetyl-glycine ethyl ester upon the pH dependence of the inactivation of pepsin. The experimental conditions for the prior mixing experiment (○—○) were those described in the legend to Fig. 3 except that the time before addition to the pepsin solution was reduced to 5 min. The relative extent of inactivation was determined as described for Fig. 4 (the values at pH 5.19 and pH 5.6 being the maxima used for the two curves). The results with no prior mixing of reagents (●—●) were obtained under the same conditions given in the legend to Fig. 4 except that the diazo compound was in a 20-fold instead of a 10-fold molar excess.

Inactivation of Pepsin by Dimethylsulfonium Phenacylide—The effect of dimethylsulfonium phenacylide on pepsin was investigated in order to secure further evidence for the nature of the reactive intermediate. As can be seen from Fig. 6, a rapid inactivation of pepsin at 37° is brought about by the reagent. Cupric ions are essential for this reaction. No inactivation occurred at 14° or in the presence of silver ions. The pH dependence is similar to that observed for diazoacetylnorleucine methyl ester. When the concentration of dimethylsulfonium phenacylide is raised to an 80-fold molar excess, inactivation of pepsin is no longer dependent upon the presence of cupric ions. No information was obtained as to the extent of incorporation of the reagent.
Diazoacetyl compounds and diazoketones both inactivate pepsin by reacting with a carboxyl group at or near the active site to form an ester. These reactions are markedly facilitated by cupric ions. Fry et al. (20) have shown that 1-diazo-4-phenylbutanone-2 reacts with the β-carboxyl group of the aspartic acid residue in the sequence Ile-Val-Asp-Thr. Bayliss and Knowles (3) showed that an aspartic acid residue had been esterified after reaction of pepsin with diazoacetylphenylalanine ethyl ester. The sequence around the substituted aspartic acid residue has not yet been reported, but from the amino acid composition of the peptide isolated, it appears likely that the same carboxyl group is involved in the reaction with both the diazoketone and the diazoacetyl derivative. This is so despite the fact that the diazo groups in these two compounds bear different steric relationships to the phenyl group.

The reasons for the speed and specificity of these Cu(II)-facilitated reactions have not been clear, but studies in several laboratories, together with the results presented in this communication, make it possible to offer plausible working hypotheses. Although it was initially thought otherwise (2), it is now quite certain that the specificity of the reaction of the diazoacetyl derivatives does not reside in the side chain of the amino acid residue. Delpierre and Fruton (4) showed that esters of diazoacetyl-glycine, diazoacetyl-n-phenylalanine, and diazoacetyl-L-phenyalanine inactivate pepsin at comparable rates. For the glycine derivative this has been confirmed in the present work and by the studies of Kozlov et al. (7). Thus, these diazo derivatives all react at or near the active site of the enzyme, but not because they are substrate analogues.

Although specificity cannot be conferred upon the reagent by the amino acid side chain alone, Delpierre and Fruton (4) showed that a measure of specificity does result from a combination of a bulky amino blocking group and a bulky amino acid side chain. They found that tosyl-L-phenylalanlyldiazomethane inactivates pepsin rapidly, whereas the D isomer reacts much more slowly. These diazo compounds have two neighboring aromatic side chains that may bind to the enzyme surface, and under these conditions, stereospecificity seems to be imposed. Delpierre and Fruton have found that tosylglycylldiazomethane inactivates pepsin at a rate intermediate between those for the L- and n-phenyalanyl derivatives.

The specificity and reactivity of the diazoacetyl reagents in the presence of Cu(II) or Ag(I) is probably a result of the formation of a metal-complexed carbene intermediate according to the following equation (14):

\[
\text{R--C--CH=NH}_2 + \text{Cu}^{2+} \rightarrow R--C--CH=\text{Cu}^+ + \text{N}_2
\]

Other routes for the decomposition of diazoketones, such as carbonium ion formation (21), are not, to our knowledge, metal-catalyzed.

The observation that a reactive intermediate is formed upon admixture of a diazoacetyl compound and Cu(II) is in harmony with such a view. An intermediate of this sort was suggested by Yates (22) as a consequence of his studies of the copper-catalyzed decomposition of diazoketones. Finally, the fact that dimethylsulfonium phenacylide also inactivates pepsin in the presence of Cu(II) lends additional support to this hypothesis. The phenacylide was chosen for test only because Trost (22) had suggested that it does form such a copper-complexed reactive intermediate.

The specificity of the reaction of this intermediate with the active site of pepsin can be explained if one assumes, as others have before, that there are at least two carboxyl groups at or near the active site (23-28). The positively charged copper complex would be bound at a negatively charged carboxyl group, whereupon reaction would occur at a neighboring protonated carboxyl group. Kinetic studies have led a number of workers to the conclusion that proteolysis by pepsin requires two carboxyl groups (28, 29). The need for a protonated carboxyl group alone, Delpierre and Fruton have shown before, that there are two neighboring aromatic side chains that may bind to the enzyme surface, and under these conditions, stereospecificity seems to be imposed. Delpierre and Fruton have found that tosylglycylldiazomethane inactivates pepsin at a rate intermediate between those for the L- and n-phenyalanyl derivatives.

The specificity of the reaction of this intermediate with the active site of pepsin can be explained if one assumes, as others have before, that there are at least two carboxyl groups at or near the active site (23-28). The positively charged copper complex would be bound at a negatively charged carboxyl group, whereupon reaction would occur at a neighboring protonated carboxyl group. Kinetic studies have led a number of workers to the conclusion that proteolysis by pepsin requires two carboxyl groups (28, 29). The need for a protonated carboxyl group is also suggested by the data in Figs. 4 and 5. In addition, carbonizes are known to substitute in a -C-OH linkage

4 Personal communication from Dr. J. S. Fruton.

5 Binding of the copper complex by the imidazole side chain of the single histidine residue in pepsin has been considered but thought to be a less likely explanation.
The existence in pepsin of carboxyl groups which are still protonated at pH 5.6 is likely from the results of Edelhoch (30), who showed that denaturation of pepsin in the neutral pH range is associated with the loss of 6 protons. Since pepsin has only one histidine which could lose protons in this region, it is logical to assign the remainder of the protons to carboxyl groups.

The participation in the catalytic action of pepsin of a carboxyl group still protonated in the range of pH 4.5 to 5.5 is not required by the kinetic experiments already cited. On the other hand, Hollands and Fruton (31) in a recent paper stress some of the anomalies connected with the action of pepsin on synthetic substrates. They point out that the pH optimum can vary widely from about pH 2 to around pH 4.5 depending upon the structure and the charge of the substrate. Their protonated substrate, Z-His-Phe-Phe-OMe, has a pH optimum above 4, suggesting that there may be several carboxyl groups near the active site which may or may not participate in catalysis depending upon the structure of the substrate. Our data suggest that if these carboxyl groups are closely spaced and have overlapping pK values ranging from around 1 to near 5, the metal-complexed carbenes would react with one that has a relatively high pK. The carboxyl group that is esterified by p-bromo-phenacyl bromide may not be any of these, since this reaction does not lead to complete inactivation of the enzyme.

The speed and specificity of the inactivation of pepsin by diazo compounds in the presence of Cu(II) is thus thought to be a result of a positive charge on the reagent in conjunction with a group which can esterify a protonated carboxyl group, coupled with the existence in the enzyme of two properly oriented carboxyl groups, one of which is protonated and one of which is not.  

Acknowledgments—We acknowledge with pleasure the excellent technical assistance of Mrs. Eugenia Greff in the performance of this work, and the help of Mr. S. Theodore Bella who carried out the microanalyses. We also wish to thank Drs. S. Moore and T. A. A. Dopheide for many helpful discussions. Our particular gratitude goes to Dr. J. S. Fruton for thought provoking conversations on the subject of this paper and for making unpublished information available to us.

REFERENCES
