work will help to further elucidate the relationships between the substrate binding sites and the heterogeneity of firefly luciferase.

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Site of Reaction of a Specific Diazo Inactivator of Pepsin*

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ABSTRACT: At pH 5.5 in the presence of Cu(II), 1-diazo-4-phenyl-2-butanone reacts with swine pepsin in 1:1 molar stoichiometry to give a catalytically inactive enzyme. The inactivator reacts with pepsin to give an ester of 1-hydroxy-4-phenyl-2-butanone with the β-carboxyl group of a uniquely reactive aspartyl residue which is believed to be at the active site. This ester as well as a related model compound hydrolyze readily at pH 8 and room temperature. Following digestion of the inactivated pepsin by swine pepsin at pH 2 several peptides containing the reacted aspartyl residue were isolated. From the sequences of these peptides the sequence around the reactive aspartyl residue is indicated to be: Ile-Val-Asp-Thr-(Gly,Thr)-Ser-Leu. This sequence is similar to that containing the aspartyl group which reacts with *N*-diazoacetyl-L-phenylalanine methyl ester (Bayliss, R. S., Knowles, J. R., and Wybrandt, G. B. (1969), *Biochem. J. 113*, 377).

Kinetic studies with pepsin indicate that at least two carboxyl groups on the enzyme are involved in catalysis (Jackson *et al.*, 1965, 1969; Clement *et al.*, 1968; Zeffren and Kaiser, 1967; Lutsenko *et al.*, 1967; Denberg *et al.*, 1968; Hollands and Fruton, 1968; Cornish-Bowden and Knowles, 1969; Lundblad and Stein, 1969). Stein and Fahrney (1968) have shown that a carboxyl group is directly involved in the pepsin-catalyzed hydrolysis of sulfite esters, and Shkarenkova *et al.* (1968) have observed that oxygen from $H_2{}^{18}O$ is rapidly incorporated into two carboxyl groups of pepsin. Erlanger and his coworkers (1965, 1966; see also Gross and Morell, 1966) have identified one uniquely reactive carboxyl group using a phenacyl halide inhibitor. However, this carboxyl is apparently not catalytically active but rather is involved in substrate binding because the modified enzyme still retains considerable catalytic activity toward hemoglobin as substrate (Erlanger *et al.*, 1967).

In an attempt to identify a carboxyl group at the active site of pepsin by means of a carboxyl-reactive reagent we chose the readily synthesized diazoketone 1-diazo-4-phenyl-2-butanone (DPB)[1] since it mimics partially the structure of phenylalanine amides which are good substrates for pepsin. DPB, in the presence of Cu(II) as catalyst, was found to react with pepsin in a 1:1 molar ratio to give a completely inactive enzyme (Hamilton *et al.*, 1967). After the work with DPB was begun, Delpierre and Fruton (1965) reported the inhibition of pepsin activity by diphenyldiazomethane. This inactivation is apparently not specific since more than 1 mole of the inhibitor reacts per mole of enzyme. Independent of our work several other groups of investigators (Rajagopalan *et al.*, 1966; Delpierre and Fruton, 1966; Ong and Perlmann, 1967; Erlanger *et al.*, 1967; Bayliss and Knowles, 1968; Kozlov *et al.*, 1967; Stepanov and Vagonova, 1968; Lundblad and Stein, 1969) have found that other diazocarbonyl compounds which have structures related to substrates of the enzymes will also inactivate pepsin.

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[1] Abbreviations used are: 1-diazo-4-phenyl-2-butanone, DPB; 1-hydroxy-4-phenyl-2-butanone, HPB; 1-acetoxy-4-phenyl-2-butanone, AcHPB; 5-dimethylaminonaphthalene-1-sulfonyl, dansyl.

We have been able to: isolate, from enzymic hydrolysis of the inactivated pepsin, peptides containing radioactivity from ^{14}C-labeled DPB; show that the inhibitor becomes attached by an ester linkage to the β-carboxyl group of an aspartyl residue; and determine the amino acid sequence in the vicinity of the reactive aspartyl group (Fry *et al.*, 1968). Knowles and his coworkers subsequently reported evidence that this aspartyl group also reacts with *N*-diazoacetyl-L-phenylalanine methyl ester (Knowles and Wybrandt, 1968; Bayliss *et al.*, 1969). Details of our work previously given only in outline, and further investigations of the sequence around the diazocarbonyl-reactive aspartyl residue, are included in this report.

Experimental Section

Materials. DPB and DPB-*2*-^{14}C were synthesized from 3-phenylpropionic acid and 3-phenylpropionic-*1*-^{14}C acid (New England Nuclear Corp.) by the procedure described by Birkofer (1947), and were purified by distillation twice at 95–100° (0.1 mm). The diazoketone is an oil at room temperature but solidifies on standing in the refrigerator. The spectral characteristics of the diazoketone are as follows: infrared (no solvent), strong absorptions at 1637 and 2100 cm^{-1}; nuclear magnetic resonance (in $CDCl_3$), τ 2.82 (singlet, relative area 5), 4.86 (singlet, relative area 1), 6.9–7.7 (complex multiplet, relative area 4); ultraviolet (in cyclohexane), maximum at 246 nm (log ϵ 4.0). The specific activity of the DPB-*2*-^{14}C used for most of the experiments was 1.50×10^8 dpm/mmole (the value of 1.62×10^8 dpm/mmole for this preparation given in a preliminary communication (Fry *et al.*, 1968) is in error). In some preliminary experiments the specific activity of the DPB-*2*-^{14}C was 3.2×10^7 dpm/mmole. 2-Diazocyclohexanone was synthesized by the procedure of Stetter and Kiehs (1965).

1-Hydroxy-4-phenyl-2-butanone (HPB) was prepared as follows from 1-chloro-4-phenyl-2-butanone (McPhee and Klingsberg, 1944). To a solution of 2.4 g of sodium in 160 ml of methanol and 2.4 ml of water was added a solution of 16 g of 1-chloro-4-phenyl-2-butanone in 80 ml of methanol. After refluxing with stirring for 2 hr, 150 ml of water was added, the pH was adjusted to 4–5 with concentrated HCl, and the reaction was refluxed for 2 additional hr. Following evaporation of the solvent under reduced pressure, the residue was washed twice with 100 ml of water, and extracted twice with 100 ml of ether. The ether extract was dried with anhydrous Na_2SO_4 and the solvent was removed by evaporation. Distillation of the residue under reduced pressure yielded 6.0 g (42% yield) of a fraction boiling at 112–114° (2 mm). On standing at room temperature the fraction solidified. Recrystallization from ether yielded white narrow plates melting at 44–45° (*Anal.* Calcd for $C_{10}H_{12}O_2$: C, 73.15; H, 7.37. Found: C, 73.41; H, 7.43). The spectral characteristics of HPB are as follows: infrared (in CCl_4), strong absorptions at 1725 and 3485 cm^{-1}; nuclear magnetic resonance (in CCl_4), τ 2.85 (singlet, relative area 5), 5.97 (singlet, relative area 2), 6.88 (singlet, relative area 1), 7.0–7.5 (multiplet, relative area 4); mass spectrum, intense peaks at *m/e* 133, 105, 91, and a weak peak at *m/e* 164 (molecular ion).

When HPB is treated with phenylhydrazine by the procedure described by McPhee and Klingsberg (1944) a phenylhydrazone is obtained (mp 111.5–112; λ_{max}^{MeOH} 274 nm (ϵ 19,300). *Anal.* Calcd for $C_{16}H_{18}N_2O$: C, 75.56; H,7.13; N, 11.01. Found: C, 75.60; H, 7.04; N, 11.10). However, when HPB is treated with 2,4-dinitrophenylhydrazine under the same conditions, or under conditions given by Brady (1931), an osazone is obtained (mp 252.5–253.5; λ_{max}^{MeOH} 393 nm. *Anal.* Calcd for $C_{22}H_{18}N_8O_8$: C, 50.58; H, 3.47; N, 21.45. Found: C, 50.69; H, 3.50; N, 21.10.)

1-Acetoxy-4-phenyl-2-butanone (AcHPB) was prepared from HPB as follows. A solution of 4 g of HPB, 30 ml of acetic anhydride, and two drops of concentrated HCl was allowed to stand at room temperature for 2 days and then refluxed for 2 hr. After evaporation of the solvent under reduced pressure, the residue solidified on cooling in ice and was recrystallized from ether. The colorless plates (4.8 g, 80% yield) melted at 43.5–44.5° and had the following characteristics. *Anal.* Calcd for $C_{12}H_{14}O_3$: C, 69.88; H, 6.84. Found: C, 70.02; H, 6.85; infrared (in CCl_4), strong absorptions at 1765, 1745, and 1230 cm^{-1} and no absorption in the 3500-cm^{-1} region; nuclear magnetic resonance (in CCl_4), τ 2.88 (singlet, relative area 5), 5.54 (singlet, relative area 2), 7.0–7.6 (multiplet, relative area 4), 7.98 (singlet, relative area 3); mass spectrum, intense peaks at *m/e* 206 (molecular ion), 146, 128, 105, and 91.

Inactivated pepsin was prepared from swine pepsin (lot no. J2211, P2589, and R3466, two- or three-times crystallized from dilute alcohol) obtained from Mann Research Laboratories. Twice-crystallized swine pepsin (lot PM 713) used for digestion of ^{14}C-labeled preparations and carboxypeptidase A (lot 7 J A) used for C-terminal determinations were purchased from Worthington Biochemical Corp. The crystalline lyophilized preparation of swine pepsinogen (lot K2298) was obtained from Mann Research Laboratories.

Dansylisoleucine was prepared by the method of Morse and Horecker (1966). Dansyl chloride and additional dansylated amino acids were purchased from the Pierce Chemical Co. Polyamide (ϵ-polycaprolactam) layer sheets were obtained from the Gallard-Schlesinger Chemical Manufacturing Corp. *N*-Acetyl-L-phenylalanyl-L-tyrosine was obtained from Cyclo Chemical Corp.

Preparation of the Inactivated Pepsin. For the experiments whose results are summarized in Table I, pepsin was dissolved in 10 ml of 0.04 M acetate buffer (pH 5.5, containing $CuSO_4$ for the reactions catalyzed by Cu(II)), and a small aliquot (*ca.* 10 μl) of an ether solution of DPB or DPB-*2*-^{14}C was added. Following incubation at 38° for the specified period of time, the solutions which initially contained DPB-*2*-^{14}C were dialyzed at 5° *vs.* 4 l. of 0.04 M buffer (pH 5.5) for at least 20 hr with at least two changes of buffer. Aliquots of these dialyzed solutions were then assayed for protein concentration, radioactivity, and catalytic activity as indicated below. Solutions which initially contained DPB were not dialyzed before assay for catalytic activity.

Large quantities (up to 1 g) of ^{14}C-labeled pepsin were prepared by reacting 5×10^{-4} M pepsin in 0.05 M acetate buffer (pH 5.5) with 5 or 10×10^{-4} M DPB-*2*-^{14}C (specific activity 1.50×10^8 dpm/mmole) in the presence of 1×10^{-4} M Cu(II) at 38° for 2–2.5 hr. After exhaustive dialysis (until no further radioactivity appeared in the dialysate) *vs.* the acetate buffer, the ^{14}C-labeled pepsin was precipitated by adding ten volumes of alcohol. After sitting overnight at 4°, the precipitated material was separated by centrifugation, washed several times with 85% alcohol, and dried *in vacuo*.

The overall recovery was *ca.* 75%. When 5×10^{-4} M DPB-*2*-^{14}C was used, 65% of the pepsin was labeled with ^{14}C, and when 10×10^{-4} M DPB-*2*-^{14}C was used, 95% of the pepsin was labeled. The labeled pepsin is only sparingly soluble in pH 5 buffers but dissolves readily in buffers of pH 6 or above.

Estimation of Pepsin and Inactivated Pepsin Concentrations. The pepsin and inactivated pepsin content of solutions was estimated from the absorbance at 278 nm assuming a molar extinction coefficient of 50,000. This is the reported extinction coefficient for pepsin solutions (Perlmann, 1966) and it was found that following inactivation with DPB or DPB-*2*-^{14}C the molar extinction coefficient at 278 nm remains the same.

Radioactivity Measurements. Standard liquid scintillation techniques using commerical instruments and Bray's solution (Bray, 1960) were employed.

Catalytic Activity of Pepsin Solutions. The catalytic activity was determined at 38° and pH 2 by the ninhydrin assay (Moore and Stein, 1948) using *N*-acetyl-L-phenylalanyl-L-tyrosine as substrate.

Identification of the Ether-Extractable Radioactive Compound Released from Inactivated Pepsin at pH 8. In the experiment summarized in Table II, 1.46×10^{-2} mmole of 95% ^{14}C-labeled pepsin was dissolved in 45 ml of 0.05 M Tris buffer (pH 8.05) and incubated at room temperature for 48 hr. After the solution was extracted three times with 50-ml quantities of ether, the combined ether layers were dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was dissolved in 30 ml of ethanol, and the solution was filtered (to remove a small amount of white solid) and counted (line 2, Table II). To the ethanol solution were added 1.86 mmoles of unlabeled HPB and a few drops of 1 N HCl. After the solution was concentrated to about 10 ml, 0.4 ml of freshly distilled phenylhydrazine was added and the solution was refluxed for 10 min. When the solution was concentrated under reduced pressure to approximately 1 ml and cooled in the refrigerator, crystals deposited. These were filtered, washed with cold ethanol, and dried *in vacuo*. An aliquot of an ethanol solution of this material was used for counting (line 3, Table II). The crystalline material was recrystallized twice from ethanol and counted as before (lines 4 and 5, Table II). Infrared and ultraviolet spectra of solutions of the recrystallized material were identical with those of authentic samples of the phenylhydrazone of HPB. The radioactivity of the phenylhydrazone reported in Table II includes a small (1–2%) quench correction.

Kinetics of Hydrolysis of AcHPB. The rate of hydrolysis of AcHPB was followed by titrating the released acetic acid with base using a Radiometer pH-Stat.

Preparation of Labeled Peptide Fragments. Extensive digestion of ^{14}C-labeled pepsin with native pepsin was performed as follows. A 172-mg sample of 95% inactivated ^{14}C-labeled pepsin (specific activity 1.43×10^8 dpm/mmole) was suspended with stirring in 3.27 ml of 0.05 M Tris-HCl buffer (pH 7.3) to give a turbid solution at pH 6.4. The small amount of insoluble material remaining after 1 hr at room temperature was dissolved by the addition of 0.1 ml of 0.1 M Tris-HCl buffer (pH 8.5), followed by an additional 40-min stirring. The pH of the resulting solution was then raised from 6.68 to 7.45 by titration with 0.4 ml of 0.1 M Tris-HCl buffer (pH 8.5). The clear slightly yellow solution obtained was allowed to stand for an additional 15 min at room temperature to ensure denaturation of the pepsin, and was then treated with 20 ml of 0.02 M HCl. The pH of the resulting suspension of precipitated protein was lowered from 2.30 to approximately 1.9 by the addition of 0.14 ml of 1 M HCl and digestion at 37° was then initiated by addition of 3.0 ml of a solution of swine pepsin (5 mg/ml in 0.01 M HCl) to the magnetically stirred suspension. Within 15 min most of the precipitate present at the beginning of the digestion had dissolved. After 4 hr an additional 3.0 ml of pepsin solution was added and the digestion was continued for an additional 14.5 hr. The resulting digest, pH 2.65, was then treated with 0.1 ml of 1 M acetate buffer (pH 5; resulting pH, 2.85) and lyophilized to dryness.

Short-term enzymic digestion of a 122-mg sample of inactivated pepsin was performed in a similar fashion except that the denatured pepsin was precipitated by the addition of 15 ml of 0.5 M formic acid to yield a suspension at pH 2.1 before digestion with native pepsin (10 mg added in 2 ml of 1 M formic acid) for 2 hr at 37°.

Fractionation of Pepsin Digests by Gel Filtration. Columns of Sephadex G-15 and Sephadex G-25 (fine bead form) were packed in 0.2 M acetic acid (Eaker, 1962) and operated under gravity flow at room temperature with 0.2 M acetic acid as eluent. The Sephadex G-25 column used in all experiments had dimensions of 1.9×243 cm and void volume of 275 ml. The Sephadex G-15 column used was 0.9×158 cm and had a void volume of 40.5 ml. Portions of fractions eluted from gel columns were assayed for ninhydrin-positive material using the method of Rosen (1957) after base hydrolysis by a method similar to that described by Hirs *et al.* (1956).

Paper Chromatography and Electrophoresis. Descending chromatography for 10–11.5 hr on Whatman No. 3MM paper was performed in 1-butanol–acetic acid–water (200:30:75, v/v) (Nolan and Margoliash, 1966).

Paper electrophoresis on Whatman No. 3MM paper in 8% formic acid (7.6 ml of 88% formic acid + 91 ml of H_2O) for 5 hr and in pyridine–acetic acid–water (1:10:289, v/v) at pH 3.6 for 4 hr (Katz *et al.*, 1959) was performed on a 30-in. water-cooled, flat-plate electrophoresis apparatus (Savant, Model FP-30A) at 3000 V. The body of the electrophoresis paper was separated from direct contact with the paper wicks dipping into the electrode troughs by two pieces (four single thicknesses) of 1-in. wide, buffer-wetted, dialysis tubing (size 20 from the Visking Co., Chicago, Ill.). This procedure (Rothman and Byrne, 1963) reduced the degree of wetting of the electrophoresis paper during extended runs.

Electrophoresis at pH 6.4 in pyridine–acetic acid–water (200:8:1800, v/v) (Offord, 1966) was performed on Whatman No. 3MM paper in a tank electrophoresis apparatus (Gilson Medical Electronics, Model D) at 115 V/cm using Varsol as the water-immiscible phase (Katz *et al.*, 1959).

Peptides were streaked on the Whatman No. 3MM paper in 0.2 M acetic acid. Radioactive bands on electrophoresis and chromatographic patterns obtained were detected by counting sections of runner strips. The center sections of the radioactive bands were eluted using 0.2 M acetic acid and the absence of significant amounts of free inhibitor in the eluates confirmed by counting ether extracts of a portion of each.

Distances of migration of bands in chromatography were measured relative to the migration of the solvent front and are designated R_{FC}. The R_{FC} value observed for marker spots of leucine in the butanol–acetic acid–water system was 0.67. Distance of migration of peptides in electrophoresis in 8%

TABLE I: Some Characteristics of the Reaction of Pepsin with DPB and DPB-*2*-^{14}C.

Concn (M × 10^4) During Incubn at pH 5.5, 38°			Length of Incubn	Inhibn of	% Pepsin with
Pepsin	DPB or DPB-*2*-^{14}C[a]	Cu(II)	(min)	Catalytic Act.	^{14}C Attached[b]
0.3	0.83	0.0	5	0	
0.3	0.83	0.0	120	40[c]	
0.3	0.83	0.1	5	84	
0.3	0.83	0.1	120	80	
0.3	0.00	1.0	120	0	
0.3	0.83[d]	0.0	120	0	
0.3	0.83[d]	1.0	120	76	
9.3	10	1.0	30	91	81
9.3	100	1.0	30	60	60

[a] Specific activity 3.2 × 10^7 dpm/mmole. [b] Assuming a 1:1 molar stoichiometry. [c] This value is not too reproducible; however, it was usually between 20 and 50. [d] During incubation the solution also contained 1.5 × 10^{-2} M *N*-acetyl-L-phenylalanyl-L-tyrosine.

formic acid was measured relative to the migration of aspartic acid and is designated R_{FE}. Distance of migration in electrophoresis at pH 3.6 is given in centimeters along with the distance of migration (toward the cathode) of a marker spot of serine. No corrections for movement due to electroosmosis were made in reporting electrophoretic migrations.

Recoveries of ^{14}C-labeled material from chromatograms and electrophoresis patterns were calculated for material eluted from the center of radioactive bands as well as that present in the corresponding region of runner strips.

Amino Acid Analyses. Peptides were hydrolyzed at 110° in evacuated, sealed tubes with 0.2 ml of twice-distilled constant-boiling HCl. Hydrolysates were evaporated to dryness at 40° under reduced pressure as recommended by Crestfield *et al.* (1963).

Amino acid analyses were performed by the method of Spackman *et al.* (1958) with a Phoenix amino acid analyzer (Model 8000 B) equipped with an accelerated flow system and with 1.0-cm path-length micro flow colorimeters (Phoenix Model 800) using loads of amino acids equivalent to 0.020–0.005 μmole of hydrolyzed peptides. The concentrations of amino acids present in amounts of less than 0.002 μmole were estimated directly from peak heights rather than by integration by the height-times-width method. Base-line stability was adequate to allow easy detection of the amino acids present in the isolated peptides in amounts as low as 0.0005 μmole.

Sequence Determinations. Conditions used for sequential analysis of peptides from the N-terminal end by the subtractive Edman method were those described by Gray (1967b). Extraction with *n*-butyl acetate (Gray, 1967b) proved adequate in the cases studied here to remove anilinothiazolinones as well as diphenylthiourea from the reaction mixture following the cyclization step. The butyl acetate extracts obtained after each cycle were evaporated in a stream of nitrogen and residues obtained, after dissolving in 0.5 ml of 95% ethanol, were added to Bray's solution for determination of ^{14}C content. Where noted, the N-terminal amino acid exposed by each stage of the degradation was determined directly following dansylation (Gray, 1967a) using chromatography on polyamide layer sheets by the method of Woods and Wang (1967). Solvent systems 1 and 2 recommended by these authors were used.

Carboxypeptidase A digestion of 6–9-nmole samples of peptides was performed using 10 μg of enzyme at 37° in a final volume of 0.11 ml of pH 8.5–8.8 morpholine buffer as described by Ambler (1967a). Released amino acids were determined directly using the amino acid analyzer.

Results

Some General Characteristics of the Inactivation. Some of the properties of the reaction of pepsin with DPB are shown by the results summarized in Table I. Although Cu(II) alone under these conditions has no effect on the enzymic catalysis,[2] it markedly catalyzes the inactivation of the enzyme by DPB. In the absence of Cu(II) there is a slow inactivation of the enzyme by DPB, but the rate is not very reproducible, possibly because the reaction is catalyzed by trace impurities of Cu(II) which are present in varying amounts. When the inactivation reaction is slow and incomplete (in the absence of added Cu(II)) then a specific substrate (*N*-acetyl-L-phenylalanyl-L-tyrosine) protects the enzyme from inactivation. This indicates that the inactivation reaction occurs at or near the active site of the enzyme.

The experiments summarized in the last two lines of Table I indicate that one molecule of DPB-*2*-^{14}C reacts with one molecule of pepsin to give a modified enzyme which is cata-

[2] V. Briones Valenty of this laboratory has recently shown that, at low ionic strength (0.01 M) and slightly higher pH's (5.7), Cu(II) does cause an inactivation of pepsin probably due to denaturation. Under the same conditions in the absence of Cu(II) the enzyme is stable. This Cu(II) effect is apparently the reason for the reported inactivation of pepsin by dimethylsulfonium phenacylide (Lundblad and Stein, 1969). We have not been able to observe any inhibition of pepsin activity attributable to reaction with dimethylsulfonium phenacylide either in the presence or absence of Cu(II). Apparently the observed inactivation (Lundblad and Stein, 1969) arose because of a slight increase in pH on addition of the basic dimethylsulfonium phenacylide to the weakly buffered pepsin solution containing Cu(II).

TABLE II: The Identity of HPB and the Radioactive Compound Released from ^{14}C-Labeled Pepsin at pH 8.

Description[a]	Total Radioactivity (dpm × 10^{-5})	Sp Act. (dpm/mmole × 10^{-5})
(1) 1.46×10^{-2} mmole of 95% labeled pepsin	20.8	1500[b]
(2) Ether-extractable radioactivity after treatment of (1) at pH 8	17.7	
(3) Phenylhydrazone prepared from (2) plus 1.86 mmoles of unlabeled HPB	11.8	8.5
(4) Recrystallized phenylhydrazone from (3)	2.25	8.6
(5) Recrystallized phenylhydrazone from (4)	0.29	8.2
(6) Expected[c] specific activity of the phenylhydrazone		8.7

[a] For details, see Experimental Section. [b] Specific activity of the DPB-*2*-^{14}*C* used to prepare the labeled pepsin. [c] Calculated from the specific activity of DPB-*2*-^{14}*C*, the known amount of unlabeled HPB added, and the total radioactivity in (2) assuming it is all due to labeled HPB.

lytically inactive toward the synthetic substrate. Experiments performed by V. Briones Valenty in this laboratory have shown that the modified enzyme is also catalytically inactive in another enzyme assay using hemoglobin as substrate (Rajagopalan *et al.*, 1966b).

Although a large number of different diazocarbonyl compounds have now been found to inactivate pepsin (for references see introduction) not all such compounds do. For example, under conditions (with Cu(II) catalysis) where DPB gives almost complete inactivation of pepsin, 2-diazocyclohexanone has no effect on the catalytic activity. This result suggests that binding at a specific site (presumably the active site) is necessary prior to the inactivation reaction. Further evidence for this is the observation that pepsinogen does not react with DPB-*2*-^{14}*C* (with Cu(II) catalysis) to give a ^{14}C-labeled pepsinogen.

Organic solvents, such as cyclohexane and ether, extract DPB almost quantitatively from aqueous solution, but extraction of the inactivated enzyme solution following incubation does not reverse the inactivation nor does it remove the radioactivity from the ^{14}C-labeled pepsin. Also, the radioactivity cannot be removed by dialysis or Sephadex chromatography. The ^{14}C-labeled pepsin can be precipitated with ethanol and washed several times with 85% ethanol with no loss in radioactivity. Following precipitation of the ^{14}C-labeled pepsin with 5 N trichloroacetic acid, the radioactivity remains in the precipitated material even after washing with organic solvents such as acetone, dioxane, cyclohexane, and ether. All these results indicate that a covalent bond is formed between the inactivator and pepsin during the inactivation reaction.

Release of Radioactivity from the ^{14}C-Labeled Pepsin and Identification of the Product. Radioactivity is not released from the ^{14}C-labeled pepsin if it is incubated at pH 5.5, 38° for extended periods of time. Also, treatment at pH 5.5, 38° for 24 hr with 0.1 M thioethanol or 0.1 M hydroxylamine followed by extended dialysis does not remove the ^{14}C. However, following incubation of the ^{14}C-labeled enzyme at pH 8 and 30° for a few hours, the radioactivity becomes ether extractable. The release of radioactivity from the ^{14}C-labeled pepsin follows first-order kinetics to well over 2 half-lives. This indicates that the ^{14}C label is attached to pepsin at only one site and not at two or more different sites. The $t_{1/2}$ for the radioactivity release at pH 8.05 (0.005 M Tris buffer) and 30.0° is 5.5 hr.

Preliminary chromatographic experiments suggested that the ether-extractable radioactive compound is HPB. The results shown in Table II verify this conclusion. The phenylhydrazone prepared from the radioactive compound plus unlabeled HPB does not change its specific activity on repeated recrystallizations. Since the observed activity of the phenylhydrazone is essentially the same as that calculated (line 6), virtually all the ether-extractable radioactivity must be present as HPB.

The Rate of Hydrolysis of AcHPB. Since it seemed possible that DPB reacts with a carboxyl group of pepsin to give an ester of HPB which then hydrolyzes at pH 8, the rate of hydrolysis of the model compound, AcHPB, at slightly basic pH's was investigated. At 30.0° and pH 8.5 to 9.6 in 5% dioxane–water the rate of hydrolysis of AcHPB is given by the expression: rate = $(58 \pm 5\ M^{-1}\ sec^{-1})$[AcHPB][$OH^-$]. Therefore, $t_{1/2}$ for the hydrolysis of AcHPB at pH 8.0 and 30.0° would be approximately 3 hr. This is comparable to the observed $t_{1/2}$ for the release of HPB from the labeled enzyme, and indicates that a reasonable structure for the inactivated pepsin is an ester of HPB with a carboxyl group of pepsin.

Isolation of Labeled Peptides after Extended Digestion. Gel filtration chromatography of the 18.5-hr digest on Sephadex G-25 gave the elution pattern shown in Figure 1. Total recovery from the column was 98% (68% in fractions 281–308). The peak at fractions 347–360 which contained 14% of the ^{14}C applied to the column is due to free inhibitor as indicated by the observations that: (a) the radioactivity in the pooled fractions was extractable with ether, and (b) an acid hydrolysate of the ether extract was devoid of amino acids.

The material in fractions 283–290 (peak I) gave a single major peak of radioactivity (eluting at 91 ml) when subjected to gel filtration on Sephadex G-15. The material in the center section of this peak (84% of the ^{14}C applied to the column) gave a single band of radioactivity (peptide I) in the successive steps: (a) chromatography (R_{FC} = 0.85; recovery, 80%), (b) formic acid electrophoresis (R_{FE} = 0.60, migration 23 cm; recovery, 88%), and (c) pH 3.6 electrophoresis (migration 7.9 cm toward cathode *vs.* 4.0 cm for serine, recovery, 84%).

The asymmetric peak of radioactivity eluted from the G-25 column in fractions 294–304 (Figure 1) was resolved into two peaks (II and III) by passage through the G-15 Sephadex column (Figure 2). Total recovery of radioactivity from the G-15 column in fractions 75–125 was 97%. Broad cuts from each peak (peak II, fractions 97–101, 24% of ^{14}C applied and peak III, fractions 104–115, 67% of ^{14}C applied)

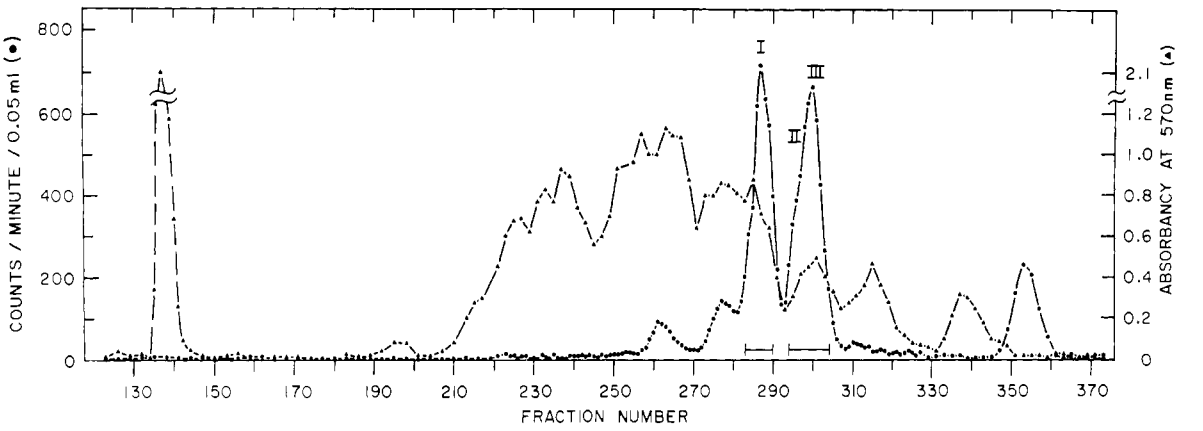

FIGURE 1: Gel filtration on Sephadex G-25 of 18.5-hr pepsin digest of inhibited pepsin. A sample containing ^{14}C equivalent to 6.4×10^5 dpm (96% of the ^{14}C present in the original digest) was applied to the column in 4.0 ml of 0.2 M acetic acid. Fractions of 2.0 ml were collected at a flow rate of 23.5 ml/hr. Absorbancy at 570 nm was measured after ninhydrin reaction with base-hydrolyzed, 0.05-ml aliquots.

were used for isolation of labeled peptides II and III, respectively, by chromatography and electrophoresis.

For peptide II single bands of radioactivity were obtained in the successive steps: (a) chromatography (R_{FC} = 0.75; recovery, 91%), (b) electrophoresis in formic acid (R_{FE} = 0.72; migration 30 cm; recovery, 30% in trailing edge, 58% in center cut), and (c) electrophoresis at pH 3.6 (migration 9.5 cm toward cathodes *vs.* 4.0 cm for serine; recovery, 89%). Amino acid analyses of portions of material eluted from the trailing edge and center section of the band in formic acid electrophoresis indicated that the same peptide was the predominant species in each.

For peptide III single peaks of radioactivity were obtained in the successive steps: (a) chromatography (R_{FC} = 0.89; recovery, 90%) and (b) electrophoresis in formic acid (R_{FE} = 0.67; migration 28 cm; recovery, 86%).

Isolation of Labeled Peptides after Short-Term Digestion. Suspension of the lyophilized 2-hr digest of inhibited pepsin in 4.0 ml of 0.2 M acetic acid, followed by centrifugation to remove the large amount of insoluble material present, yielded a supernatant solution (3.81 ml) containing approximately 64% of the ^{14}C initially present in the digest. Gel filtration of the soluble material on Sephadex G-25 gave an elution pattern (Figure 3) which contained two broad, asymmetric peaks of radioactivity (peaks A and B). The positions of elution of these peak fractions indicated that they contained labeled peptide fragments which were larger than those present in the peak fractions obtained by gel filtration of the long-term digests (peaks I, II, and III in Figure 1). The radioactivity recovered in peak A (fractions 236–255, 1.09×10^5 dpm) and peak B (fractions 265–280, 1.28×10^5 dpm) accounted for 34 and 40% respectively, of the ^{14}C applied to the column. The peak of radioactivity at fractions 349–360 was due to free inhibitor as determined by ether extraction.

Passage of material from peak A through the column of Sephadex G-15 yielded a slightly asymmetric peak of radioactivity eluting at 71 ml (91% recovery) with no significant separation of labeled components. Chromatography of this material gave two broad radioactive bands with approximate R_{FC} values of 0.67 and 0.83. Recovery of ^{14}C from the major faster migrating band was 49% (4.19×10^4 dpm). Electrophoresis of the material from this band in 8% formic acid yielded a broad distribution of radioactivity (Figure 4a). The portion of the electrophoresis pattern eluted with an approximate R_{FE} = 0.42 contained 40% of the ^{14}C (1.35×10^4 dpm) in the sample initially applied to the paper. Electrophoresis of the eluate at pH 3.6 (Figure 4b) yielded a single major band of radioactivity (distance of migration 3.8 cm toward cathode *vs.* 3.6 cm for serine). Total recovery of ^{14}C in the three cuts indicated was 90%, 67% (7400 dpm) in the center fraction. The material in the center fraction has been designated peptide A.

Gel filtration on G-15 Sephadex, paper chromatography, and electrophoresis of material in peak B (Figure 3) indicated the presence in this peak of several radioactively labeled peptides including peptide I. Sufficient purification was not obtained, however, to allow sequence determination of these peptides.

Determination of the Sequence of the Labeled Peptides. Amino acid composition of the purified peptides and results

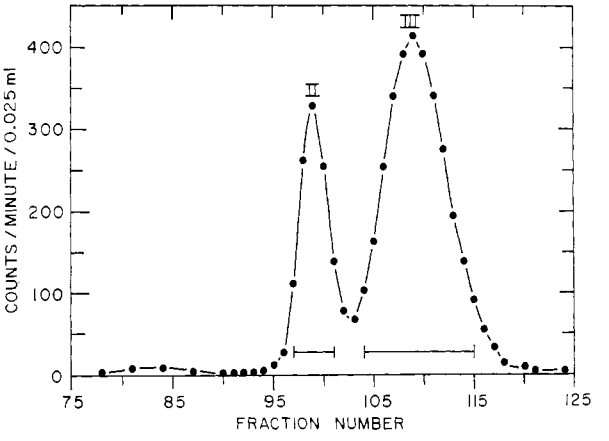

FIGURE 2: Gel filtration on Sephadex G-15 of pooled, lyophilized fractions 294–304 (peak II, III) from gel filtration of 18.5-hr digest on Sephadex G-25. A sample containing ^{14}C equivalent to 2.04×10^5 dpm was applied to the column in 1.01 ml of 0.2 M acetic acid. Fractions of 0.93 ml were eluted at a flow rate of 5.74 ml/hr.

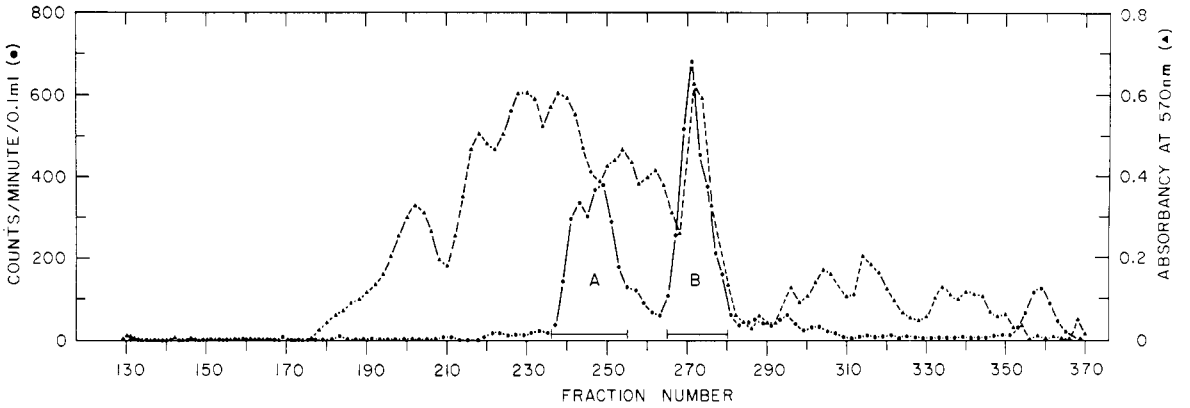

FIGURE 3: Gel filtration on Sephadex G-25 of 2-hr pepsin digest of inhibited pepsin. A sample containing ^{14}C equivalent to 3.24×10^5 dpm was applied to the column. Fractions of 2.01 ml were collected at a flow rate of 25.2 ml/hr. Absorbancy at 570 nm was measured after ninhydrin reaction with base-hydrolyzed, 0.05-ml aliquots.

of the subtractive Edman degradations are given below. Initial amino acid analysis of each peptide was performed after 69-hr hydrolysis while residues remaining from each stage of the Edman degradation were hydrolyzed 39 hr before analysis. No corrections were made for destruction of labile amino acids (Ser and Thr) during acid hydrolysis. In presenting results of the Edman degradations values for the residue removed at each stage have been indicated in boldface type and the values used in calculating recoveries have been placed in parentheses. The percentage recovery at each stage was calculated from the amount of peptide submitted to degradation at that stage. The original peptides were neutral in electrophoresis at pH 6.4 indicating the absence of a free β-carboxyl group in Asp.

Peptide I:	Ile	-	Val	-	(Asp inhibitor)	-	Thr
Composition	0.9		1.0		1.0		0.9
Step 1 (82%)	**<0.1**		1.0		(1.0)		0.9
Step 2 (80%)			**<0.1**		(1.0)		0.9
Step 3 (69%)			<0.1		**0.1**		(1.0)

The sequence was confirmed by the dansyl Edman procedure. Amino acid analysis of a portion of the residue remaining after step 3 without prior acid hydrolysis indicated the amount of free Thr expected if Thr is C terminal. Measurements for ^{14}C on the butyl acetate layers obtained at each stage of the Edman degradation indicated inhibitor was removed from the peptide in step 3, *i.e.*, with the Asp residue. Ratio μmoles of inhibitor (^{14}C)/μmoles of Asp = 0.99.

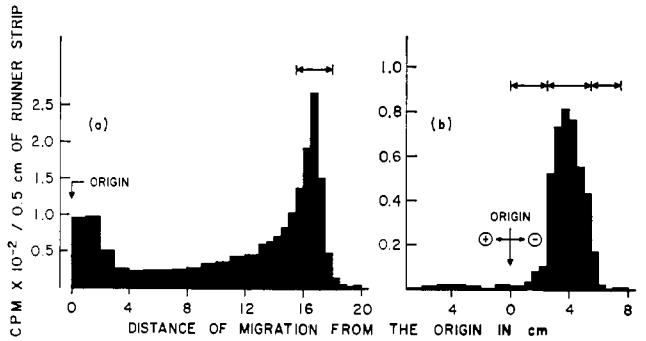

FIGURE 4: Distribution of ^{14}C in runner strips obtained in purification of ^{14}C-labeled material from faster migrating radioactive band in chromatography of peak A by (a) electrophoresis in 8% formic acid for 5 hr and (b) electrophoresis at pH 3.6 for 4 hr.

Peptide II:	Val	-	(Asp inhibitor)	-	Thr
Composition	1.0		0.9		0.9
Step 1 (75%)	**<0.1**		(1.0)		0.9
Step 2 (76%)	0.1		**0.1**		(1.0)

This peptide was slightly impure (approximately 10% Gly, Ala, Leu, and Phe present). The inhibitor was removed from the peptide in step 2 with Asp, and free Thr released. Ratio μmoles of inhibitor (^{14}C)/μmoles of Asp = 0.99.

Peptide III:	Ile	-	Val	-	(Asp inhibitor)
Composition	0.9		1.0		1.0
Step 1 (77%)	**<0.1**		1.0		(1.0)
Step 2 (79%)			**0.1**		(1.0)

Ratio μmoles of inhibitor (^{14}C)/μmoles of Asp = 1.00. The ratio of inhibitor:Asp in the residue following step 2 was 1.03. Treatment of a portion of the residue from step 2 with 0.5 ml of 5% trimethylamine for 30 min at room temperature followed by evaporation of the solvent *in vacuo* over H_2SO_4 liberated Asp (approximately 70% yield) as indicated by amino acid analysis performed without prior acid hydrolysis.

Peptide A:	Ile	-	Val	-	(Asp inhibitor)	-	(Thr, Gly)	-	Ser	-	Leu
Composition	1.0		1.1		1.2		2.2		1.2		1.0		1.1
Step 1	**<0.1**		0.9		(1.0)		1.7		1.0		0.7		0.8

(Residue from Edman was hydrolyzed 69 hr before analysis.)

Carboxypeptidase A (4.5 hr, 37°) Leu, 0.8; Ser, 0.5; Thr, 0.2

Carboxypeptidase A (16 hr, 37°) Leu, 0.9; Ser, 0.7; Thr, 0.4

Although the relative amount of Thr increased from 16 to 37% in carboxypeptidase A digestions for 4.5 and 16 hr, respectively, the absolute amounts of Thr released in each case, 0.001 and 0.002 μmole, were not sufficient to firmly establish Thr as the third amino acid from the C-terminal end of the peptide (see Discussion). Ratio μmoles of inhibitor (^{14}C)/μmoles of Asp = 1.02.

Rate of Hydrolysis of ^{14}C Label from Peptides. In order to ensure that the ^{14}C label is attached to the peptides in the same way as to pepsin, the relative rates of hydrolysis of the ^{14}C label from pepsin and the three peptides were determined. The relative rates are: ^{14}C-labeled pepsin, 1.0; peptide I, 2.2; peptide II, 3.2; peptide III, 0.4. Although the data are probably not reliable to better than 10%, they clearly show that the ^{14}C label is released from the peptides at a rate comparable to the rate of release from pepsin. Thus, presumably the bonding of the ^{14}C label has not changed during the digestion of the labeled pepsin to yield the labeled peptides.

Discussion

The results reported here are consistent with the conclusion that DPB inactivates pepsin by reacting to give an ester of HPB with the β-carboxyl group of only one specific aspartyl residue. The results with peptides I, II, and III indicate the amino acid sequence in the immediate vicinity of the reactive aspartyl residue to be Ile-Val-Asp-Thr. The amino acid composition of peptide A, as well as identification of Ile as the N-terminal residue and Ser-Leu as the C-terminal sequence, indicate that this peptide is probably from the same site in the enzyme as that from which peptides I–III were derived and thus has the sequence: Ile-Val-Asp-Thr-(Gly,Thr)-Ser-Leu. Although not enough purified peptide A was obtained to allow direct determination for tryptophan, the absence of the latter from the interior of this peptide may be concluded from recent results of Dopheide and Jones (1968) on the sequence around the four tryptophan residues in pepsin. It seems likely, therefore, that the aspartyl residue in the sequence: Ile-Val-Asp-Thr-Gly-Thr-Ser, shown by Knowles and coworkers (Knowles and Wybrandt, 1968; Bayliss *et al.*, 1969) to be reactive toward *N*-diazo[1-^{14}C]acetyl-L-phenylalanine methyl ester, is the same one that reacts with DPB, as reported here and in a prior communication (Fry *et al.*, 1968). However, the position of this aspartyl residue in pepsin is not known; the above sequence is not present in any of the reported partial sequences of pepsin (Dopheide *et al.*, 1967; Dopheide and Jones, 1968; Perham and Jones, 1967; Foltman and Hartley, 1967; Koehn and Perlmann, 1968; Matveeva *et al.*, 1968; Ostoslavskaya *et al.*, 1968; Vakhitova *et al.*, 1968; Kostka *et al.*, 1969).

Evidence that DPB reacts at only one site in the pepsin molecule includes: (1) the stoichiometry of the inactivation; loss of catalytic activity correlates directly with the reaction of 1 mole of DPB with 1 mole of pepsin to give a modified enzyme, and pepsin will not react with more DPB even in the presence of a large excess; (2) the kinetics of ^{14}C release from ^{14}C-labeled pepsin at pH 8; the release follows first-order kinetics to over 80% reaction; and (3) the amino acid sequence data: the yields, compositions, and sequences of the ^{14}C-labeled peptides isolated are consistent with only one uniquely reactive site.

That the inactivated pepsin is an ester of HPB with the β-carboxyl group of a uniquely reactive aspartyl residue is indicated by the neutrality of peptides I–III at pH 6.4, and removal of the ^{14}C-label during Edman degradations with the removal of the aspartic acid residue. The comparable rates of hydrolysis of the model compound AcHPB and inhibited pepsin further indicate attachment of the inhibitor by an ester linkage. The surprising ease of hydrolysis of AcHPB and inactivated pepsin may be due to (1) the electrostatic effect of the ketonic group making the alcoholate a better leaving group, and (2) intramolecular participation in the hydrolysis by the hydrate of the ketonic group. Large accelerations due to intramolecular participation in the rate of hydrolysis of closely related ketonic or aldehydic esters have been observed (Newman and Hishida, 1962; Bender and Silver, 1962).

The evidence, that the aspartyl residue which reacts with DPB is directly involved in the mechanism of the enzymic catalysis, although not conclusive, is indicative. Observations which indicate this are: (1) substrates of pepsin protect it from inactivation by DPB, (2) the modified enzyme is completely inactive in at least two assays, using *N*-acetyl-L-phenylalanyl-L-tyrosine and hemoglobin as substrates, and (3) pepsinogen does not react with DPB.

As indicated in the introduction, kinetic studies have shown that at least two different carboxyl groups of pepsin are involved in the enzymic catalysis. If either of these is the one which reacts with DPB then it is probably the one with the higher p*K*; diazo compounds are known to react more readily with un-ionized carboxylic acids than with carboxylate groups.

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