Enzymatic and Chemical Properties of an Endopeptidase from the Larva of the Hornet *Vespa crabro*†

Klaus-Dieter Jany,* Harald Haug,‡ Gerhard Pfleiderer, and Jacob Ishay§

ABSTRACT: An endopeptidase from the larvae of the hornet *Vespa crabro* has been purified to homogeneity. The enzyme has been characterized with respect to molecular weight, amino acid composition, and amino- and carboxyl-terminal sequences. The catalytic properties of the hornet protease are similar to those of bovine chymotrypsin with respect to inactivation by phenylmethanesulfonyl fluoride and carbobenzoxyphenylalanine chloro ketone and preferential peptide bond cleavage at aromatic amino acid residues. In contrast to bovine chymotrypsin, the hornet protease is not inhibited by the basic pancreatic Kunitz inhibitor, soybean inhibitor, or chicken ovomucoid. The molecular weight, as determined by several independent methods, was found to be 14 500. The protease is a single-chain protein containing two disulfide bonds. The terminal sequences are: NH_2-Ile-Val-Gly-Gly-Ile-Asp . . . . . Gly-Lys-Tyr-Pro-Tyr-Gln-Val-Ser-Leu-Arg-COOH.

Even though most living animals are insects, the investigation of the proteolytic enzymes for these species has not progressed very far in comparison with microbial and vertebrate endopeptidases. Insect proteases have been only poorly characterized, often in crude extracts. Most of these enzymes have been termed trypsin- or chymotrypsin-like on the basis of the hydrolyses of specific synthetic substrates and as the result of inactivation by distinct active-site-directed inhibitors (Law et al., 1977). Recently, a few insect endopeptidases have been purified to a homogeneous stage and characterized. These, mostly trypsin-like enzymes, have been compared in evolutionary terms with mammalian serine proteases (Kafatos et al., 1967; Zwilling, 1968; Kramer et al., 1973; Miller et al., 1974). The catalytic properties, molecular weights, amino acid compositions, and sequence analyses indicate homologies with the endopeptidases of bacteria, invertebrates, and vertebrates, affirming a divergent evolution of these enzymes from a common ancestral gene (deHaen et al., 1975). A remarkable fact is that during evolution the length of the polypeptide chain remained nearly constant with a molecular weight of ca. 25 000. We have, previously, reported the isolation of a chymotrypsin-like protease with a molecular weight of 13 800 (VOP II) from the larvae of the hornet *Vespa orientalis* (Jany et al., 1974a,b). The low molecular weight of this hornet protease may be an indication (but not proof) of converging evolution. Since such an enzyme should not be unique and in order to obtain further information on the digestive enzymes of insects, we have investigated the endopeptidases of the larvae of the hornet *Vespa crabro*. Three endopeptidases—one with tryptic and two with chymotryptic properties—were isolated from the midguts (Jany, unpublished). This species allows the isolation of more material for sequence analysis than is available from *Vespa orientalis*.

The present paper describes the chemical and enzymatic properties of the chymotrypsin-like protease (henceforth, VCP II) which is distinguished from the other chymotryptic endopeptidases by its low molecular weight.

Experimental Section

Chemicals. Reagents were obtained as follows: The inhibitors $PhCH_2SO_3F$,[1] Z-PheCH_2Cl, Tos-PheCH_2Cl, chicken ovomucoid, soybean inhibitor, protein standards for molecular weight determination, and the reagents for dansyl-Edman degradation were from Serva, Heidelberg. Enzyme substrates and all other chemicals were from E. Merck, Darmstadt. Polyamide plates 1700 F were from Schleicher & Schüll, Dassel. The basic pancreatic Kunitz inhibitor was a gift from Bayer, Leverkusen and Glut-$(Ala)_3$-pNan, Glut-Ala-Gly-Phe-pNan were a gift from Dr. Femfert, Universität Bochum. [U-^{14}C]phenylalanine was from Amersham Buchler, Braunschweig. Z-[^{14}C]PheCH_2Cl was synthesized according to Shaw (1967) (mp 100–103 °C, lit. 102–103 °C, specific radioactivity 0.16 mCi/mmol). The B chain of oxidized insulin was prepared according to Fittkau (1963). The chain was tested for homogeneity by amino acid analysis and dansylation. Pyridine, butyl acetate, and *N*-ethylmorpholine were distilled after refluxing with ninhydrin. The larvae of *Vespa crabro* were obtained from the Institut für Bienenkunde, Oberursel.

Enzyme Assay and Purification. The enzyme activity was routinely measured with glutaryl-L-phenylalanine-*p*-nitroanilide (Glut-Phe-pNan) (Erlanger et al., 1964). The test mixture contained 500 μL of 4.2 mM substrate in 0.2 M Tris-HCl[1] buffer, pH 8.2 (25 °C). The reaction was started with 25 μL of enzyme solution, and the liberation of *p*-nitroaniline was monitored at 405 nm. An absorption coefficient (405 nm) of 9620 M^{-1} cm^{-1} was used; the unit of activity is expressed in terms of nanokatals (nkat). One katal (kat) is defined as the amount of enzyme which catalyzes the hydrol-

† From the Universität Stuttgart, Institut für Organische Chemie, Biochemie und Isotopenforschung, Pfaffenwaldring 55, D-7000 Stuttgart 80, Federal Republic of Germany. *Received April 12, 1978; revised manuscript received July 14, 1978.* This work was supported in part by the Deutsche Forschungsgemeinschaft.

‡ Taken in part from the Ph.D. Thesis of H. Haug.

§ Present address: Tel-Aviv University, Sackler School of Medicine, Department of Physiology and Pharmacology, Tel-Aviv, Israel.

[1] Abbreviations used are: Glut, glutaryl; Ac, acetyl; pNan, 4-nitroanilide; Pth, phenylthiohydantoin; EAP, mixture of monoethanolamine phosphate and triethanolamine phosphate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Tos-PheCH_2Cl, 1-tosylamidophenylalanine chloro ketone; $PhCH_2SO_3F$, phenylmethanesulfonyl fluoride; DFP, diisopropyl fluorophosphate; CM, reduced and *S*-carboxymethylated; CM-Z-Phe-CH_2Cl VCP II, carbobenzoxyphenylalanine chloro ketone inhibited, reduced, and *S*-carboxymethylated hornet protease II; VOP II, *Vespa orientalis* protease II.

0006-2960/78/0417-4675$01.00/0

ysis of 1 mol of substrate/s under the assay conditions. The term nanokatal (nkat) follows the usual convention. The kinetic parameters were calculated from the initial velocity of six substrate concentrations by the procedure of Cornish-Bowden (1975) on a Wang 2200 computer. The hydrolysis of azocasein, casein, or hemoglobin was assayed at pH 7.8 (Jany, 1976a). The protease VCP II was purified from the larval midguts as described previously (Jany et al., 1974a), except that CM-Sephadex C-25 was replaced by CM-Sepharose Cl-6B and Sephadex G-75 by Sephadex G-50.

Protein Determination. Protein concentrations were determined by the microbiuret procedure using LAB-Trol (DADE, Miami) as standard or by quantitative amino acid analyses.

Polyacrylamide Gel Electrophoresis. Gel electrophoresis at pH 4.3 (Reisfeld et al., 1962) was carried out on a micro scale for which protein staining, as well as enzyme specific staining, was performed and described previously (Dahlmann & Jany, 1975). Determination of the molecular weight by gel electrophoresis without sodium dodecyl sulfate was performed on gels of increasing acrylamide concentrations (12.5–25%) according to Hedrick and Smith (1968) using bovine trypsin (M_r 23 500), myoglobin (17 200), ribonuclease A (13 400), and cytochrome *c* (12 400) as standards.

Sodium dodecyl sulfate gel electrophoresis was carried out by the method of Laemmli (1970). The molecular weight of VCP II was determined from the linear calibration curve (Weber & Osborn, 1969) obtained from bovine serum albumin (M_r 68 000), pepsin (35 500), chymotrypsinogen (25 500), soybean inhibitor (21 500), myoglobin, and cytochrome *c*.

Gel Filtration. Molecular weight and Stokes radius were also estimated by gel filtration on Sephadex G-75 (1.5 × 100 cm) equilibrated in 50 mM (pH 7.8) Tris-HCl containing 0.2 or 1 M NaCl (Andrews, 1970; Siegel & Monty, 1966).

Active-Site Titration. Active-site titration was performed with *p*-nitrophenyl trimethylacetate [S_0 4.2 mM, 50 mM Tris-HCl (pH 8.0), 2% acetonitrile (v/v) at 405 nm] (Kézdy & Kaiser, 1970). The active-site concentration was calculated from the amount of nitrophenol liberated in the "burst" reaction using the absorption coefficient (405 nm) of 16 240 M^{-1} cm^{-1}.

Inhibition Studies. The inactivation by the synthetic inhibitors was investigated by incubation of the enzyme (18 μM) with various amounts of the inhibitors (0.6–5.0 mM) in 0.1 M phosphate buffer (pH 7.4) at 25 °C. Aliquots of the mixture were removed at regular intervals and assayed for residual activity. VCP II used for active-site labeling or sequence analysis was incubated with a 15 M excess of Z-[^{14}C]-PheCH_2Cl until no activity could be detected. Possible traces of active enzyme were removed by affinity chromatography on phenylbutylamine-Sepharose 4B, and the inhibited protease was rechromatographed on Sephadex G-50 (120 × 3 cm) equilibrated in 30% acetic acid.

Naturally occurring endopeptidase inhibitors were mixed with VCP II at molar ratios of 10:1, 50:1, and 100:1, and the residual activity was determined after 30- and 60-min incubation intervals.

Cleavage Specificity. The cleavage specificity was determined with the B chain of oxidized insulin. The B chain (20 mg) was digested at a substrate to enzyme ratio of 100:1 (w/w) in 0.1 M *N*-ethylmorpholine acetate (pH 8.0) at 35 °C for 5 and 20 h. The hydrolysis was stopped by boiling the digest for 10 min after which the solution was lyophilized. The digest was dissolved in 2 mL of 50% formic acid, and 1.95 mL was chromatographed on a Dowex 50W-X4 column (0.6 × 1.20 cm). Pyridine acetate buffer (pH 2.9, 180 mL, 50 mM) was passed through the column, followed by a linear gradient consisting of 500 mL of starting buffer and 500 mL of 2 M pyridine acetate (pH 5.0). The flow rate was 35 mL/h, and 2.5-mL fractions were collected, the peptides were detected by the manual ninhydrin method (Moore & Stein, 1954), and fractions containing peptides were lyophilized and subjected to amino acid analysis.

Carboxymethylation and Citraconylation. The Z-[^{14}C]-PheCH_2Cl-inhibited protease was reduced with dithioerythritol and carboxylmethylated with iodoacetic acid as described by Crestfield et al. (1963). As the resulting S-alkylated protease is completely insoluble in buffers used for tryptic digestion, a citraconylation was performed, for which 6.8 μmoles of the protein was dissolved in 10 mL of 10 M urea and then the pH was adjusted to 8.5. A 1000-fold molar excess of citraconic anhydride was gradually added (10-μL charges) during which the pH was kept constant by the addition of 4 M NaOH. The soluble protein was desalted by gel filtration on Sephadex G-25 and then lyophilized. Prior to the tryptic digestion, a second citraconylation was carried out, but urea was omitted.

Tryptic Digestion. The digestion of the 6.2 μmoles of citraconylated CM-Z-[^{14}C]PheCH_2Cl protease was initiated by the addition of 2% (w/w) specially purified trypsin (Jany et al., 1976b); after 1 h, a second aliquot of trypsin was added. The digestion was performed in 0.1 M *N*-ethylmorpholine acetate (pH 8.5) at 35 °C for 3 h, after which the mixture was lyophilized.

Isolation of the Peptides. The tryptic digest was dissolved in 6 mL of 70% formic acid and chromatographed on a Sephadex G-50 column (3 × 120 cm) equilibrated with 30% acetic acid. Further purification of the fragment T4-2 was achieved by thin-layer chromatography on purified cellulose plates (Chen, 1976) using the solvent system 1-butanol–acetic acid–water (4:1:1, v/v). The peptides, located by staining a guide strip with ninhydrin, were recovered by elution of the corresponding cellulose section with 30% acetic acid.

Amino Acid Analysis. The amino acid analyses were carried out according to Spackman (1967) on a Biotronik analyzer LC 6000. The amino acid composition of VCP II is derived from a triplicate analysis of 0.2 mg of protein hydrolyzed in 6 N HCl at 108 °C for 24, 48, 72, and 96 h. The values for serine and threonine are extrapolated to zero time. Isoleucine, valine, and leucine are the average from the 96-h hydrolysis. Cysteine and methionine were determined after performic acid oxidation (Hirs, 1967) as cysteic acid and methionine sulfone, respectively. Tryptophan was determined after hydrolysis in 3 N mercaptoethanesulfonic acid (Penke et al., 1974).

End Group Analysis. NH_2-terminal residues were determined after dansylation (Weiner et al., 1972) on 5-cm-square polyamide sheets (Woods & Wang, 1967). COOH-terminal residues were evaluated by digestion with DFP-treated carboxypeptidase B or a mixture of carboxypeptidases A and B. Twenty to fifty nanomoles of protein/peptide was dissolved in 500 μL of *N*-ethylmorpholine acetate (pH 8); the exopeptidase was added as specified (Figure 2, Table III). At different times, 100-μL samples were withdrawn, and the reaction was stopped by boiling. The solution was evaporated to dryness, and the released amino acids were analyzed on the amino acid analyzer. Blanks were performed on solutions identical to the digestion mixtures without carboxypeptidase or the protein/peptide (vice versa). Norleucine was used throughout as internal standard.

Sequence Analysis. The dansyl-Edman degradation was carried out on 30 nmol of peptide using essentially the same micro procedure described by Chen (1976). After each deg-

TABLE I: Purification of the Hornet Protease VCP II.

	total act. (nkat)[a]	total protein (mg)	sp act. (nkat/mg)	purification (fold)	yield (%)
extraction of 140-g midguts	1114.4	8578	0.13	1	100
DEAE-Sephadex A-50	1084.6	2266	0.48	4	97
CM-Sepharose Cl-6B	805.2	153	5.26	41	72
phenylbutylamine-Sepharose 4B	678.4	113	5.98	46	61
Sephadex G-50	592.4	95	6.21	48	53

[a] Activity determined with Glut-Phe-pNan as substrate.

radation step, the Pth derivatives were further analyzed by thin-layer chromatography (Asx/Glx) or on the amino acid analyzer after back-hydrolysis with 6 N HCl containing 0.1% $SnCl_2$ (Mendez & Lai, 1975). Automated Edman degradation was performed in a Beckman 890 C sequencer using polybrene and a special Quadrol program (Ponstingl, personal communication). Pth-amino acids were identified by thin-layer chromatography and high pressure liquid chromatography.

Results

Purification and Homogeneity. The procedure for the isolation of the endopeptidases VCP II, starting from 140 g of larval midguts (wet weight), is summarized in Table I. The enzyme preparation lacks trypsin, carboxypeptidase A and B, and aminopeptidase activities, even when large amounts of enzyme were added to the test mixture for long incubation times. Analytical disc electrophoresis of VCP II in 15% gels at pH 4.3 shows a single protein band; this band coincided with that found by means of enzyme specific staining (Figure 1). The purity was further proven by dansylation, which yielded only isoleucine and the dipeptide Ile-Val.

Molecular Weight Determination. Analytical gel filtration on Sephadex G-75 yielded a molecular weight of 14 300, which is similar to that previously determined for protease II of *V. orientalis* (VOP II, M_r 13 800, Jany et al., 1974a). Since this method can give an anomalously low molecular weight for certain proteases (Voordouw et al., 1974), several methods (I–IV) were employed to more firmly establish the molecular weight of VCP II.

(I) Active-site labeling of VCP II with Z-[^{14}C]PheCH$_2$Cl was performed as outlined under the Experimental Section. After complete inactivation, 66.2 nmol of the inhibitor was incorporated per milligram of protein. The molecular weight can be calculated to 15 100, assuming a 1:1 stoichiometry. The determination of the active-site concentration of VCP II could not be performed with *p*-nitrophenyl acetate because a very rapid turnover of the acyl enzyme follows the "burst" reaction. When VCP II (0.28 mg) is reacted with *p*-nitrophenyl trimethylacetate, the steady-state reaction is depressed and the "burst" can be separated. Extrapolation to zero time gives a "burst" of 0.3175 absorbance unit which corresponds to 19.55 nmol of active-site equivalents. Thus, the molecular weight can be calculated to be 14 600 for a 1:1 stoichiometric formation of the acyl enzyme.

(II) Carboxypeptidase A digestion of the citraconylated CM-Z-PheCH$_2$Cl protease II released no amino acid residues. However, carboxypeptidase B rapidly liberated arginine and leucine (Figure 2). If one assumes a molecular weight of 14 500, the amount of protein used for digestion correlates well with the quantity of arginine or leucine liberated.

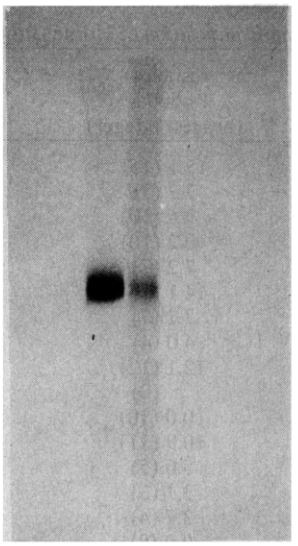

FIGURE 1: Analytical disc gel electrophoresis at pH 4.3. Direction of migration is from anode (top) to cathode (bottom). Left, 20 μg of protein applied, stained with Coomassie blue for protein. Right side, 5 μg of protein applied and enzyme-specific staining with Ac-Tyr-pNan.

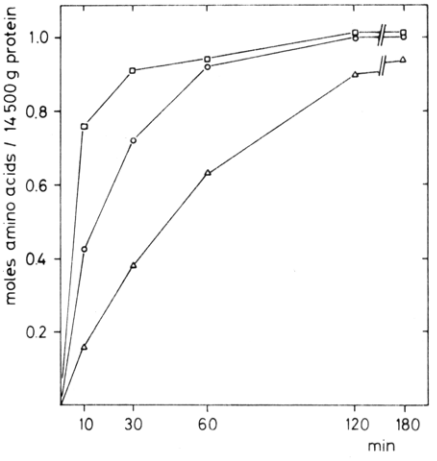

FIGURE 2: Carboxypeptidase B digestion of citraconylated CM-Z-PheCH$_2$Cl-inhibited VCP II: (□–□) arginine; (○–○) leucine; (△–△) serine. 0.7 mg of protein was digested with carboxypeptidase B (30:1, w/w) in *N*-ethylmorpholine acetate (pH 8.0) at 30 °C.

(III) Electrophoresis at pH 4.3 of VCP II and standard proteins on gels of increasing polyacrylamide concentrations (12.5–25%) according to Hedrick and Smith (1968) revealed a molecular weight of 14 100.

(IV) Sodium dodecyl sulfate gel electrophoresis of the native or of the Z-[^{14}C]PheCH$_2$Cl-labeled protease in the presence or absence of 2-mercaptoethanol showed three bands. The major band corresponded to a molecular weight of 25 000 and the two minor bands to 14 000 and 55 000.

When using labeled protease all bands showed radioactivity. The amino acid analysis of the bands revealed no significant deviations of the amino acid composition from the material used for electrophoresis. The low molecular weight is further supported by amino acid analysis (Table II) and by fingerprints. Mapping the tryptic digest of CM-Z-PheCH$_2$Cl VCP II yielded approximately 18 peptides, whereas in the digest of the citraconylated protein six peptides could be detected. Further, calculation of the diffusion coefficient and Stokes radius (Siegel & Monty, 1966; Ackers, 1964) on the basis of the gel-filtration data resulted in values of 125 $\mu m^2\ s^{-1}$ and 1.67 nm, respectively. These values are similar to those of VOP

TABLE II: Amino Acid Composition of Hornet Protease II.

amino acid	residues/ 14 500 g[a] (nearest integer)	integral × mol wt of residue	VOP II[e]	SGPA[f]	SGPB[f]	α-LP[f]	Chy A[g]
Asp	15.1 (15)	1.725	13	16	17	15	22
Thr	7.2 (7)	701	6	22	28	18	22
Ser	9.6 (10)	870	9	22	22	20	27
Glu	10.2 (10)	1.290	10	8	4	13	15
Pro	7.2 (7)	679	6	4	5	4	9
Gly	14.1 (14)	798	13	32	33	32	23
Ala	7.2 (7)	497	6	19	15	24	22
½-Cys[b]	4.0 (4)	408	4	4	4	6	10
Val	12.1 (12)	1.188	12	12	13	19	23
Met	(0)		0	1	2	2	2
Ile	10.0 (10)	1.130	10	9	7	8	10
Leu	10.9 (11)	1.243	10	10	7	10	19
Tyr	5.0 (5)	815	4	8	10	4	4
Phe	3.1 (3)	441	3	5	5	6	6
His	3.7 (4)	548	4	3	2	1	2
Lys	9.0 (9)	1.152	8	0	1	2	14
Arg	6.2 (6)	936	5	7	8	12	3
Trp[c]	2.1 (2)	368	2	1	1	2	8
total	136	14 807[d]					

[a] Average of three analyses. [b] Determined as cysteic acid after performic acid oxidation. [c] Determined after mercaptoethanesulfonic acid hydrolysis. [d] Molecular weight corrected for one molecule of water. [e] Jany et al. (1974a). [f] Jurásek et al. (1976). [g] Hartley (1964).

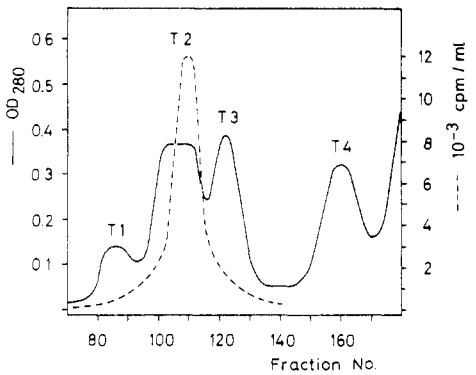

FIGURE 3: Chromatography of the tryptic digest citraconylated CM-Z-[^{14}C]PheCH$_2$Cl VCP II. 87 mg of the digest dissolved in 6 mL of 70% formic acid was applied on the column (120 × 3 cm). The column was developed with 30% acetic acid, and 4.5-mL fractions were collected.

TABLE III: Amino Acid Composition and Carboxypeptidases A and B Digestion of Peptide T4-2.

amino acid[a] comp			nmol of amino acids released[b] 10	30	60	180	240
Ser	1.06	(1)		0.2	0.7	1.7	3.1
Glu	1.04	(1)					
Pro	1.12	(1)					
Gly	1.21	(1)					
Val	1.06	(1)			0.9	1.5	2.5
Leu	0.92	(1)	1.3	3.0	4.0	4.5	4.9
Tyr	1.84	(2)					
Lys	0.88	(1)					
Arg	1.0	(1)	5.9	6.1	6.3	6.3	6.3

[a] The amount of arginine was taken as one residue. [b] Peptide T4-2 was digested with the exopeptidases at a ratio of 40:1 (w/w) at pH 8.0. Each aliquot taken from the digestion mixture corresponds to 6 nmol.

II (D = 131 μm^2 s^{-1}; r = 1.62 nm) obtained by ultracentrifuge techniques.

Amino Acid Composition. The amino acid composition of VCP II is given in Table II and compared with some other serine proteases. VCP II is devoid of methionine, and probably the protease is not a glycoprotein, since glucosamine or galactosamine could not be found. Titration of VCP II with Nbs_2 in the presence of protein denaturants revealed no free sulfhydryl groups. Apparently, all cysteine residues are fully incorporated in disulfide bonds.

Amino- and Carboxyl-Terminal Sequences. In the native as well as in the CM-Z-PheCH$_2$Cl protease, isoleucine was identified as the unique NH_2-terminal residue, suggesting that the hornet protease II is a single-chain protein. This supposition is further affirmed by the automated Edman degradation of 100 nmol of CM-Z-PheCH$_2$ClVCP II, which revealed the NH_2-terminal sequence Ile-Val-Gly-Gly-Ile-Asp-; no other amino acids could be detected in the individual steps.

In order to obtain the carboxyl-terminal peptides, the tryptic digest of the citraconylated CM-Z-[^{14}C]PheCH$_2$ClVCP II was chromatographed on Sephadex G-50 which resulted in four peaks (Figure 3). The fractions corresponding to peak T4 were pooled and taken to dryness. An aliquot was further purified by thin-layer chromatography, yielding the arginine peptides T4-1 and T4-2. The amino acid composition and the results from the carboxypeptidases A and B digestion of T4-2 are given in Table III. The time course of the liberation of amino acids from T4-2 by the carboxypeptidase digestion indicates the sequence -Val-Ser-Leu-Arg, positioning this peptide at the carboxyl terminus (Figure 3). The dansyl-Edman degradation of 30 nmol of peptide T4-2 reveals -Gly-Lys-Tyr-Pro-Tyr-Gln-Val-Ser-Leu-Arg as the C-terminal sequence of VCP II.

Enzymatic Properties. The effect of pH on the hydrolysis of synthetic substrates (Ac-Tyr-pNan, Glut-Phe-pNan) was investigated using the following buffers: 0.1 M sodium phosphate–citric acid (pH 5.0–7.2), 0.1 M Tris-HCl (pH 7.2–8.4), and 0.1 M EAP–NaOH (pH 8.0–11). The pH optimum was found to be between pH 7.8 and 8.4. VCP II is quite stable at protein concentrations less than 0.8 mg/mL within the pH range of 6–9, but below pH 4 the protease II gradually loses its activity, presumably due to irreversible acid denaturation.

TABLE IV: Catalytic Parameter of Hornet Protease VCP II and Bovine Chymotrypsin.

substrate: -*p*-nitroanilide	VCP II			chymotrypsin A			
	k_{cat}/K_m (M^{-1} s^{-1})	$K_m^{app} \times 10^4$ (M)	$k_{cat} \times 10^2$ (s^{-1})	K_{cat}/K_m (M^{-1} s^{-1})	$K_m^{app} \times 10^4$ (M)	$k_{cat} \times 10^2$ (s^{-1})	S^a (mM)
Glut-Ala-Ala (1)	0			0			4
Glut-Ala-Ala-Ala- (1)	0.7	28.7	0.2	0			0.18–6.9
Glut-Leu- (1)	1.1	37.8	0.4	0.04			1.28–20.6
Glut-Tyr-	343.6	9.3	31.9	105.4	8.4	8.9	0.03–8.7
Ac-Tyr-	19.4	179.6	19.5	50.9	12.4	9.8	
Glut-Phe-	76.3	6.9	5.3	45.6	4.6	2.1	0.07–10.8
Glut-Phe- (2)	38.5	32.2	12.4				0.34–20.2
Gly-Phe- (1)	24.2	9.8	2.4	15.1	10.3	1.6	0.13–5.8
Glut-Gly-Phe-	344.7	1.9	6.8	51.9	7.0	3.6	0.03–7.6
Glut-Ala-Gly-Phe-	561.8	3.8	21.3	3948.9	12.9	551.0	0.03–5.5
casein[b]	0.95			20.88			
hemoglobin[b]	0.48			8.11			

[a] The catalytic constants were determined in 0.2 M Tris-HCl (pH 8.2) using the given substrate concentrations. Test mixture containing (1) 5% (v/v) dimethylformamide, (2) 10% dimethylformamide. [b] Activity expressed as mol of tyrosine.

TABLE V: Analyses of Peptides from VCP II Digest of the Oxidized B Chain of Insulin.

amino acid	amino acid composition[a]											
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
$CysO_3H$	1.1 (1)	1.0 (1)	1.1 (1)	0.4 (1)	1.1 (1)		1.2 (1)	1.0 (1)				
Asp								1.1 (1)		1.1 (1)		
Thr									0.9 (1)			1.0 (1)
Ser	1.0 (1)	1.0 (1)	0.9 (1)					0.8 (1)				
Glu	1.2 (1)		1.1 (1)	1.3 (1)	1.0 (1)	1.0 (1)	1.1 (1)	1.3 (1)		1.0 (1)		
Pro									1.2 (1)			1.1 (1)
Gly	1.2 (1)	1.0 (1)	1.1 (1)	0.7	1.9 (2)		1.8 (2)	1.2 (1)				
Ala	0.9 (1)		0.9 (1)	1.0 (1)		1.0 (1)			1.0 (1)			1.0 (1)
Val	1.0 (1)		1.0 (1)	1.2 (1)	0.9 (1)	0.9 (1)	1.0 (1)	1.2 (1)		0.9 (1)		
Leu	1.9 (2)	1.2 (1)	2.0 (2)	1.3 (1)	1.0 (1)	1.0 (1)	1.2 (1)	2.0 (1)		1.1 (1)		
Tyr			0.7 (1)	3.2 (3)		0.9 (1)					1.2 (1)	1.1 (1)
Phe			3.0 (3)	0.3	1.0 (1)		1.7 (2)	1.1 (1)		1.1 (1)	1.1 (1)	
His	1.1 (1)	1.0 (1)	1.0 (1)					1.7 (2)		1.0 (1)		
Lys									1.1 (1)			1.1 (1)
Arg					1.0 (1)		0.9 (1)					
res[b]	7–15	7–11	7–16 25	12–15 16, 26	17–24	12–16	17–25	1–11	27–30	1–6	25–26	26–30
yield			10%	22%	46%	40%	72%		48%	50%		

[a] 24-h hydrolysis; uncorrected for decomposition during hydrolysis. [b] Residues are as shown in Figure 5.

The catalytic properties of VCP II for the hydrolysis of some substrates are compared with those of chymotrypsin A in Table IV. The cleavage specifity of VCP II was determined from the 5- and 20-h digest of the B chain of oxidized bovine insulin. The fractionation of the digests was accomplished on Dowex 50 W X-4. The 5-h digest yielded free phenylalanine, tyrosine, and the five following peptide fragments: Phe(1)-Tyr(16), Leu(17)-Phe(24, 25), Phe(25)-Ala(30), Thr(27)-Ala(30), and Phe(25)-Tyr(26).

The effluent curve obtained from the 20-h digest is shown in Figure 4. The peaks (1–12) indicated by a solid line were pooled and subjected to amino acid analysis. The amino acid composition of the peptides isolated from this digest is given in Table V. Nearly all splitting points producing these fragments could be readily identified. Peptide 4 contained free tyrosine. It is therefore possible that this fragment was derived from the residues 12–16 or 12–17. From the recoveries of the peptides, it can be seen that major cleavages occurred at the aromatic amino acid residues. The Leu(11)-Val(12) bond is completely hydrolyzed within 20 h. On the other hand, the cleavage of the same bond between positions 17 and 18 occurred only to a very small extent, probably due to the rapid splitting at Tyr(16). Obviously, a free amino group prevents

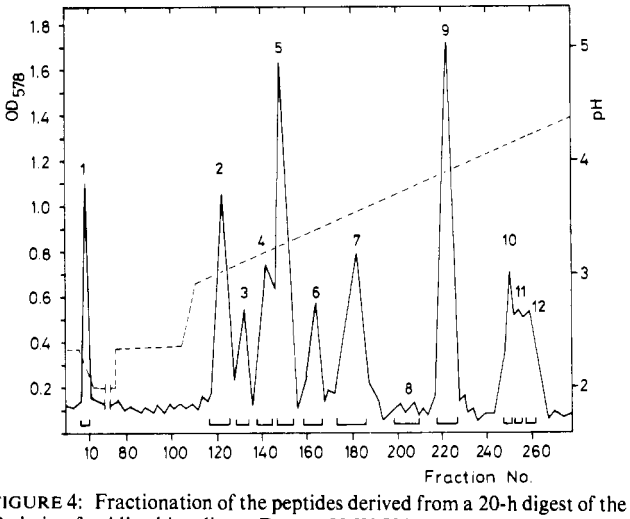

FIGURE 4: Fractionation of the peptides derived from a 20-h digest of the B chain of oxidized insulin on Dowex 50 W-X4.

the enzymatic attack, which also explains the observation that the NH_2-terminal phenylalanine is not cleaved. In Figure 5 the peptides obtained from the 20-h digest are aligned with the sequence of the B chain of oxidized insulin, and the cleavage

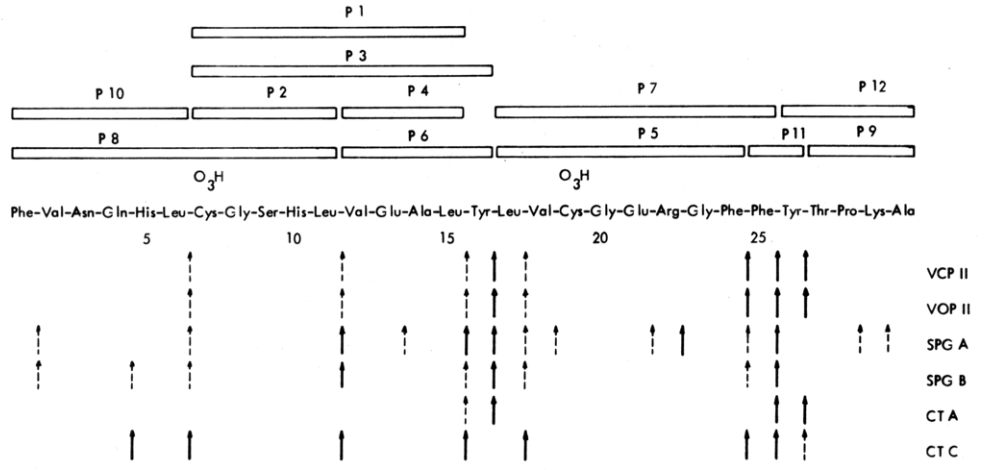

FIGURE 5: Summary of the results on the cleavage of the B chain of oxidized insulin. P1–P12 corresponds to the peptides isolated from the 20-h digest. Major cleavages are indicated by solid arrows and minor ones by dashed arrows. Abbreviations used: VOP II, *Vespa orientalis* protease II (Jany, unpublished); SGPA, *Streptomyces griseus* protease A (Johnson & Smillie, 1971); SGP B, *Str. griseus* protease B (Narashi & Yoda, 1973); CT A, chymotrypsin A (Sanger & Tuppy, 1951); CT C, chymotrypsin C (Folk & Cole, 1965).

TABLE VI: Inactivation of Hornet Protease VCP II[a] by Active-Site-Directed Inhibitors.

inhibitor	$[I] \times 10^4$ (M)	$10^4 k_{obsd}$[b] (s^{-1})	$k_{obsd}/[I]$	$K_i \times 10^4$ (M)	$k_2 \times 10^4$ (s^{-1})
$PhCH_2SO_3F$[c]	1.25	79.8	64.1		
	0.62	42.0	67.7		
	0.31	24.1	76.8		
Tos-Phe-CH_2Cl[d]	5.0	10.4	2.1		
	2.5	9.4	3.7		
	1.2	7.3	5.8	0.82	12.2
Z-PheCH_2-Cl[d]	2.5	12.45	4.9		

[a] VCP II concentration: 1.78×10^{-5} M, enzyme assays were performed using Glut-Phe-pNan as substrate, 0.1 M phosphate buffer (pH 7.4). Inhibition constants were calculated by the method of Kitz & Wilson (1962). [b] Average of three runs. Controls containing no inhibitor but the solvent concentration as indicated exhibited no decrease in activity within 60 min. [c] Ten percent (v/v) 2-propanol. [d] Twenty percent (v/v) methanol.

specifity of VCP II is compared with that of some other endopeptidases.

The active-site-directed inhibitors $PhCH_2SO_3F$ and Tos-PheCH_2Cl (Z-PheCH_2Cl) inactivate chymotrypsin by reacting with the serine and histidine residue at the active center (Shaw, 1967). These compounds also rapidly inhibited VCP II. The inactivation follows pseudo-first-order kinetics; the inhibition constants are listed in Table VI. To demonstrate that Tos-PheCH_2Cl also alkylates a histidine residue in VCP II, amino acid analysis of the inhibited protease was performed. It was found to contain one histidine residue per mole less than the native protein. Further, the amino acid analysis of the performic acid oxidized inhibited protease showed one 3-carboxymethylhistidine residue [0.78 mol of CM-His/mol, based on 90% of the ninhydrin color value of glycine (Crestfield et al., 1963)]. Some naturally occurring protease inhibitors, including the basic pancreatic Kunitz inhibitor, soybean inhibitor, and chicken ovomucoid, were also tested for their ability to inactivate VCP II. Only the soybean inhibitor (1:50 molar ratios) showed an inhibition of 20% within 2 h, whereas the other inhibitors totally failed to inactivate VCP II, even at enzyme–inhibitor ratios of 1:100.

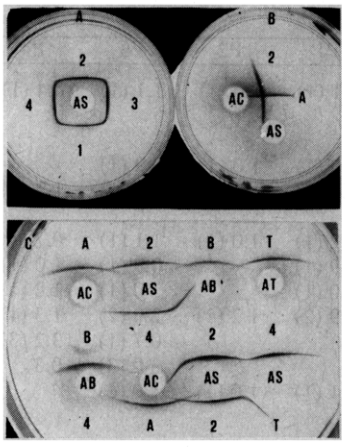

FIGURE 6: Double immunodiffusion, 1% agarose in 50 mM Tris-HCl (pH 8.0). (A) Hornet proteases VCP II (1,2) and VOP II (3,4) against VOP II antiserum (AS); 20 μL of AS in center well; (1) 4.8, (2) 9.6, (3) 6.2, and (4) 12.4 μg in outer wells. (B) Hornet protease VCP II (2) against VOP II antiserum (AS) and bovine chymotrypsin A (A, 15 μg) against chymotrypsin antiserum (AC, 40 μL). (C) Immunodiffusion of hornet proteases VCP II (2), VOP II (4), honey bee chymotrypsin-like protease B (B, 8 μg), chymotrypsin (A), and trypsin (T, 10 μg) against the corresponding antisera. (AB) protease B antiserum, 60 μL, (AT) trypsin-antiserum, 40 μL.

Immunological Studies. In Ouchterlony gel diffusion, the antiserum against VOP II gives a strong precipitin line against VCP II. This line could be stained for enzymatic activity with Ac-Tyr-pNan, demonstrating that the antiserum reacted with VCP II itself. As shown in Figure 6A, the completely closed precipitin lines between VCP II and VOP II indicate immunological identity and a high degree of structural similarities of both proteases. The hornet proteases II are related to bovine chymotrypsin and protease B from the honey bee (*Apia mellifica*) (Giebel et al., 1971) in their enzymatic properties. Therefore, antibodies to each enzyme and trypsin were prepared to determine whether any immunological (structural) relationships exist between these enzymes. However, no cross-reactions between the hornet proteases and the other enzymes could be detected (Figure 6B, C).

Discussion

The endopeptidase VCP II belongs to the group of serine proteases in which a serine and a histidine residue participate

in the catalytic mechanism, as shown by the inactivation of $PhCH_2SO_3F$ and the chloro ketones derived from phenylalanine. However, this mechanism has independently evolved in the trypsin-related endopeptidases and in the subtilisins. The restricted cleavage specificity and the amino acid composition of VCP II indicate that the hornet protease II should be included in the chymotrypsin family rather than in the subtilisins, as the latter lack disulfide bonds (Ottesen & Svendsen, 1970). Also, the amino-terminal sequence of VCP II Ile-Val-Gly-Gly- is identical to that commonly found in trypsin-related endopeptidases. Among these enzymes, molecular weights were found in the range of 19 000–26 000. Therefore, a striking feature of VCP II is its molecular weight of about 14 500. Evaluation of the molecular weight on the basis of the active enzyme, i.e., active-site labeling or titration, results in values (15 100 and 14 600), which are in fair agreement with that obtained by gel filtration. The low molecular weight is further supported by the quantitative C-terminal amino acid determination for which 1 mol of arginine is released per mole of enzyme, assuming a molecular weight of 14 500. No reasonable explanation for the quantity of arginine can be based upon a molecular weight of 25 000 (indicated by sodium dodecyl sulfate gel electrophoresis). The amino acid composition, based on the low molecular weight, shows that VCP II contains nine lysine and six arginine residues. An extended tryptic digest of the carboxymethylated protein should produce 15–16 peptides, whereas the citraconylated one should give only six fragments, if the assumed molecular weight is correct. The maps of these digests showed the expected peptides, which is indicative of the low molecular weight of VCP II. Furthermore, the values calculated for the Stokes radius (1.67 nm) and the diffusion coefficient (125 $\mu m^2 s^{-1}$) agree well with those obtained by ultracentifuge studies of protease II from *V. orientalis* (Jany et al., 1974a).

Sodium dodecyl sulfate gel electrophoresis of the homogeneous enzyme preparation (native VCP II, Z-[^{14}C]PheCH_2Cl VCP II, CM-Z[^{14}C]PheCH_2Cl VCP II) gave protein bands corresponding to molecular weights of 14 000, 25 000, and 55 000. The latter values are much higher than the molecular weight determined by the other methods. All bands also showed radioactivity, and the higher molecular weight protein species could not be distinguished by the amino acid analysis from the lower one. Therefore, we conclude that the latter components result from aggregation phenomena despite the relatively stringent conditions used for dissociation. Sodium dodecyl sulfate gel electrophoresis of native endopeptidases often results in protein species of lower molecular weights, due to autocatalytic degradation during preincubation in the denaturation buffer (Weber et al., 1972). However, in the case of the inhibited proteases, the smallest protein should not be a proteolytic fragment, as the material was chromatographed on Sephadex G-50 in 30% acetic acid prior to electrophoresis. Moreover, this material showed only isoleucine as NH_2-terminal residues.

The sequences Gly-Lys-Tyr-Pro-Tyr-Gln-Val-Ser-Leu-Arg-COOH do not correspond to any of the known sequences of the trypsin-related endopeptidases. The C-terminal arginine residue may suggest that VCP II is a tryptic fragment of the higher molecular weight protease (VCP I) present only in small amounts in the midgut. However, all attempts to convert this protease into VCP II by addition of crude larval extract, the trypsin-like protease VCP III or bovine trypsin were unsuccessful (unpublished results). It may still be possible that VCP II is the active fragment of another peptidase, which is completely degraded during the purification procedure. Nevertheless, the hornet protease VCP II shares more structural similarities with the chymoelastases A and B (SGP A and B) from *Streptomyces griseus* than with bovine chymotrypsin.

The serine-proteases SGP A and B, thought to be primitive endopeptidases (James, 1976), also have a significantly lower molecular weight (about 18 500). Like VCP II, these enzymes are single-chain proteins and both contain two disulfide bridges (Jurásek et al., 1974). In contrast, bovine chymotrypsin is composed of three polypeptide chains and has five disulfide bonds (Blow, 1971).

Despite these differences, the chymoelastases A and B are homologous with chymotrypsin (Jurásek et al., 1974) and also have similarities in their analytic properties.

The hornet protease II shows a preference for the cleavage of peptide linkages involving the carboxyl group of aromatic amino acids. Furthermore, VCP II also readily cleaves at aliphatic leucine residues, but VCP II has a more restricted specifity than the *Streptomyces* enzymes.

The kinetic constants for the hydrolysis of Glut-Phe-pNan by chymotrypsin and VCP II are identical, but their activities differ markedly against protein substrates. These substrates were hydrolyzed rather slowly by VCP II.

These results, together with the inactivation by active-site-directed inhibitors, indicate that the domain of the active site of VCP II should be similar to the above-mentioned endopeptidases, but differences may exist at the substrate binding site, as has been shown for SGP B (Gertler, 1974).

Immunological techniques were often used to estimate the degree of relationship between different proteins. The cross-reaction of starfish trypsin (Arnon & Neurath, 1969) or cocoonase (Berger & Kafatos, 1971) with antibodies toward bovine trypsin has further affirmed the homology between these enzymes that had been concluded from molecular weight, amino acid composition, and catalytic properties (Winter & Neurath, 1970; Kafatos et al., 1967). In contrast, the hornet protease VCP II cross-reacts only with antibodies against the corresponding enzyme (VOP II) from *V. orientalis,* but not with those against chymotrypsin, trypsin, or honey bee chymotrypsin-like protease B, which indicate differences in those regions of the peptide chain that determine antigeneity. Although the hornet protease VCP II differs remarkably from chymotrypsin with respect to molecular weight, amino acid composition, and its lack of inactivation by naturally occurring protease inhibitors, only further sequence analysis will reveal whether a relationship exists to the trypsin-related endopeptidases or whether this protease has evolved independently to the same catalytic mechanism.

Acknowledgments

We are indebted to Dr. Koeniger, Institut für Bienenkunde, Oberursel, for collecting the larvae of *Vespa crabro* and to Mrs. Galaboff for excellent technical assistance. Further, we are grateful to Dr. Ponstingl, Deutsches Krebsforschungszentraum (DKFZ), Heidelberg, for using his sequencer equipment and for generous advice.

References

Ackers, G. K. (1964) *Biochemistry 3,* 723–730.
Andrews, P. (1970) *Methods Biochem. Anal. 18,* 3–55.
Arnon, R., & Neurath, H. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64,* 1323–1328.
Berger, E., & Kafatos, F. C. (1971) *Immunochemistry 8,* 391.
Blow, D. M. (1971) *Enzymes, 3rd Ed. 3,* 185–212.
Chen, R. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357,* 837–886.
Cornish-Bowden, A. (1975) *Biochem. J. 149,* 305–312.

Crestfield, A. M., Stein, W. H., & Moore, S. (1963) *J. Biol. Chem. 238,* 2413–2419 (appendix 2420).
Dahlmann, B., & Jany, K. D. (1975) *J. Chromatogr. 110,* 174–177.
De Haën, C., Neurath, H., & Teller, D. C. (1975) *J. Mol. Biol. 92,* 225–259.
Erlanger, B. F., Cooper, A. G., & Bendich, A. J. (1964) *Biochemistry 3,* 1880–1883.
Fittkau, S. (1963) *Naturwissenschaften 50,* 522.
Folk, J. E., & Cole, P. W. (1965) *J. Biol. Chem. 240,* 193–197.
Gertler, A. (1974) *FEBS Lett. 43,* 81–85.
Giebel, W., Zwilling, R., & Pfleiderer, G. (1971) *Comp. Biochem. Physiol. 38,* 197–210.
Hartley, B. S. (1964) *Nature (London) 201,* 1284–1287.
Hedrick, J. L., & Smith, A. J (1968) *Arch. Biochem. Biophys. 126,* 155–164.
Hirs, C. H. W. (1967) *Methods Enzymol. 11,* 197–199.
James, M. N. G. (1976) in Proteolysis and Physiological Regulation (Ribbons, D. W., & Brew, K., Eds.), Vol. 11, p 125, Academic Press, New York, San Francisco, and London.
Jany, K. D. (1976a) *Comp. Biochem. Physiol. B 53,* 31–38.
Jany, K. D., Pfleiderer G., & Molitoris, H. P. (1974a) *Eur. J. Biochem. 42,* 419–428.
Jany, K. D., Tabatabai, S., & Pfleiderer, G. (1974b), *FEBS Lett. 48,* 53–55.
Jany, K. D., Keil, W., Meyer, H., & Kiltz, H. H. (1976b) *Biochim. Biophys. Acta 453,* 62–66.
Johnson, P., & Smillie, L. B. (1971) *Can. J. Biochem. 49,* 548–562.
Jurásek, L., Carpenter, M. R., Smillie, L. B., Gertler, A., Levy, S., & Ericsson, L. H. (1974) *Biochem. Biophys. Res. Commun. 61,* 1095–1100.
Jurásek, L., Olafson, P., Johnson, P., & Smillie, L. B. (1976) in Proteolysis and Physiological Regulation, (Ribbons, D. W., & Brew, K., Eds.), Vol. 11, p 93, Academic Press, New York, San Francisco, & London.
Kafatos, F. C., Tartakoff, A. M., & Law, J. H. (1967) *J. Biol. Chem. 242,* 1477–1487.
Kézdy, F. J., & Kaiser, E. T. (1970) *Methods Enzymol. 19,* 3–27.
Kitz, R., & Wilson, J. B. (1962) *J. Biol. Chem. 237,* 3245–3249.
Kramer, K. J., Folsted, R. L., & Law, J. H. (1973) *J. Biol. Chem. 248,* 3021–3028.
Laemmli, U. K. (1970) *Nature (London) 227,* 680–685.
Law, J. H., Dunn, P. E., & Kramer, K. J. (1977) *Adv. Enzymol. 45,* 389–425.
Mendez, E., & Lai, C. Y. (1975) *Anal. Biochem. 68,* 47–53.
Miller, J. W., Kramer, K. J., & Law, J. H. (1974) *Comp. Biochem. Physiol. B 48,* 117–129.
Moore, S., & Stein, W. (1954), *J. Biol. Chem. 211,* 907–913.
Narahashi, Y., & Yoda, K. (1973) *J. Biochem. (Tokyo) 73,* 831–841.
Neurath, H., Walsh, K. A., & Gertler, A. (1974) in Metabolic Interconversion of Enzymes, p 301, Springer Verlag, New York, N.Y.
Ottesen, M., & Svendsen, J. (1970) *Methods Enzymol. 19,* 199–215.
Penke, B., Ferenczi, R., & Kovacs, K., (1974), *Anal. Biochem. 60,* 45–50.
Reisfeld, R. A., Lewis, U. I., & Williams, D. E. (1962), *Nature (London) 195,* 281–283.
Sanger, H., & Tuppy, H. (1951) *Biochem. J. 49,* 481.
Shaw, E. (1967) *Methods Enzymol. 11,* 677–686.
Siegel, L. W., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112,* 346–362.
Spackman, D. H. (1967) *Methods Enzymol. 11,* 3–27.
Voordouw, G., Gaucher, G. M., & Roche, R. S. (1974) *Biochem. Biophys. Res. Commun. 58,* 8–12.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244,* 4406–4412.
Weber, K., Pringle, J. R., & Osborn, M. (1972) *Methods Enzymol. 26,* 3–27.
Weiner, A. M., Platt, T., & Weber, K. (1972) *J. Biol. Chem. 247,* 3242–3251.
Winter, W. P., & Neurath, H. (1970) *Biochemistry 9,* 4673–4679.
Woods, K. R., & Wang, K. T. (1967) *Biochim. Biophys. Acta 113,* 366–369.
Zwilling, R. (1968) *Hoppe-Seyler's Z. Physiol. Chem. 349,* 326–332.
Zwilling, R., Neurath, H., Ericsson, L. H. & Enfield, D. L. (1975) *FEBS Lett. 60,* 247–249.