[CONTRIBUTION FROM THE RESEARCH DIVISION, ARMOUR AND COMPANY]

The Mode of Action of Pancreatic Carboxypeptidase. I. Optical and Structural Specificity[1]

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The acyl moiety of a dipeptide exerts a pronounced influence on the susceptibility of the peptide to pancreatic carboxypeptidase. There are substantial differences in hydrolytic rates among different N-acyl derivatives of L-tyrosine. The diastereoisomers D-leucyl-L-tyrosine and L-leucyl-L-tyrosine are hydrolyzed at equal rates. However, acylation of L-leucyl-L-tyrosine results in a 50- to 200-fold increase in hydrolytic rate, whereas acylation of D-leucyl-L-tyrosine results in almost complete resistance to carboxypeptidase action. It is concluded that not only the C-terminal residue of a polypeptide but also the adjacent amino acid residue must be oriented stereospecifically at the active site of the enzyme for optimal activation. The optical specificity of the leucylglycine-splitting enzyme found in certain carboxypeptidase preparations is different from that of carboxypeptidase. The results of these studies are correlated with known specificity requirements of carboxypeptidase and other exopeptidases.

Introduction

The rates at which synthetic substrates are hydrolyzed by CPase[3] depend chiefly on the nature of the C-terminal residue. Optimal structural requirements are fulfilled by tripeptide analogs containing aromatic amino acid residues of the L-configuration in the C-terminal position.[4] Acylated amino acids, *e.g.*, chloroacetyl- and acetyl-L-phenylalanine, have lower rates of activation than acylated dipeptides as well as lower affinities for the enzyme.[5] Thus the secondary peptide group is required for optimal activity.

Dipeptides have been considered poor substrates of CPase, inasmuch as the few which had been studied were split either at extremely low rates or not at all.[6,7] Gly-L-Tyr, for example, was split about ten-thousand times more slowly than its carbobenzyloxy derivative.[7]

Our interest in the specificity requirements of CPase arose from the observation that diastereoisomers of Leu-L-Tyr were hydrolyzed at approximately equal rates[1] and that these rates were, unexpectedly, much greater than those of glycyl peptides. Moreover, a study of the action of CPase on dipeptides and their N-acyl derivatives seemed valuable in view of the widespread use of this enzyme to determine C-terminal residues and amino acid sequences of proteins and polypeptides.[8] The results of these studies have provided further insight into the mode of action of this enzyme.

In this paper the hydrolytic susceptibility of dipeptides and the stereochemical specificity of CPase toward diastereoisomers of Leu-L-Tyr and their N-acyl derivatives will be considered.[9]

(1) Certain portions of this work have been reported. *Federation Proc.*, **13**, 326 (1954).

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(3) The following abbreviations are used: CPase, pancreatic carboxypeptidase; C-terminal and C-terminus, pertaining to that end of a peptide chain with a free carboxyl group; N-terminal and N-terminus, pertaining to that end of a peptide chain with a free α-amino group; amino acid residues of peptides, abbreviated according to E. Brand and J. T. Edsall, *Ann. Rev. Biochem.*, **16**, 224 (1947); Cbz, carbobenzyloxy.

(4) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(5) J. E. Snoke and H. Neurath, *J. Biol. Chem.*, **181**, 789 (1949).

(6) M. Bergmann, *Science*, **79**, 439 (1934).

(7) K. Hofmann and M. Bergmann, *J. Biol. Chem.*, **134**, 225 (1940).

(8) P. Desnuelle, *Advances in Enzymol.*, **14**, 261 (1953).

(9) The relationship between affinity and hydrolytic susceptibility of CPase substrates is considered in the following paper, S. Yanari and M. A. Mitz, THIS JOURNAL, **79**, 1154 (1957).

Experimental

Enzyme Preparations.—CPase preparations recrystallized four times (Armour—Lot No. 372121) and six times (Armour—Lot No. 381169-6X) were prepared by accepted methods.[10,11] A third preparation, designated in Table IV as "Enzyme C," was made by Anson's procedure.[12]

The six-times recrystallized CPase was used in all experiments except the study of an aminopeptidase-like comtaminant in the other two CPase preparations. The specific activity of this preparation was at least 90% of values given in the literature.[11,13]

Substrates.—Compounds other than those discussed below were purchased from the following firms: Hoffmann–La Roche and Co., Mann Research Laboratories, Inc., General Biochemicals, Inc., and H. M. Chemical Co.

The diastereoisomers D-Leu-L-Tyr and L-Leu-L-Tyr were obtained from the first three of the above-mentioned sources. Data from quantitative paper chromatography[14] and ultraviolet spectrophotometry indicated that preparations of L-Leu-L-Tyr contained about 0.2 mole of free tyrosine per mole of dipeptide.[15] Cbz-L-Ser-L-Tyr and L-Ser-L-Tyr were gifts from Dr. K. Hofmann of the University of Pittsburgh.

N-Acyl derivatives of the diastereoisomers were prepared as follows: chloroacetyl and acetyl derivatives by acylating with the anhydrides,[16] the glycyl derivative by treating the chloroacetyl derivative with 28% ammonia at room temperature for three days, and carbobenzyloxy derivatives by the conventional procedure.

Methods.—The enzymatic reactions were carried out at 25° in 0.1 *M* LiCl and either 0.05 *M* phosphate buffer at *p*H 7.5 or 0.05 *M* aminomethylpropanediol buffer at *p*H 9.0. A modification[17] of the quantitative ninhydrin assay of Moore and Stein[18] was employed to determine the extent of hydrolysis. Since the reaction rates of dipeptides with ninhydrin vary considerably,[17] the assays were run under carefully controlled conditions to yield reproducible results. Calculation of the data was similar to that of Schwartz and Engel.[19] Results are expressed in terms of per cent. hydrolysis or in values proportional to apparent proteolytic coefficients.[4] Formol titration was used during the initial phase of this work. The products of hydrolysis and the purity of the substrates were determined by paper chromatography.

(10) F. W. Putnam and H. Neurath, *J. Biol. Chem.*, **166**, 603 (1946).

(11) N. Neurath, E. Elkins and S. Kaufman, *ibid.*, **170**, 221 (1947)

(12) M. L. Anson, *J. Gen. Physiol.*, **20**, 663 (1937).

(13) E. Elkins-Kaufman and H. Neurath, *J. Biol. Chem.*, **175**, 893 (1948).

(14) We are grateful to W. F. White and A. M. Gross for the determination of the purity of many compounds.

(15) Column chromatography on a cellulose ion exchanger demonstrated that even a "chromatographically pure" preparation of Leu-L-Tyr (Mann Research Laboratories) contained about 0.1 mole of tyrosine per mole of dipeptide. (Unpublished results.)

(16) S. M. Birnbaum, L. Levintow, B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **194**, 455 (1952).

(17) S. Yanari, *ibid.*, **220**, 683 (1956).

(18) S. Moore and W. H. Stein, *ibid.*, **176**, 367 (1948).

(19) T. B. Schwartz and F. L. Engel, *ibid.*, **184**, 197 (1950).

Results

The Hydrolysis of Dipeptides by CPase.—In Table I the hydrolytic rates of several dipeptides, one tripeptide, and the reference compound, Cbz-Gly-L-Phe, are compared. Much higher enzyme concentrations and longer incubation periods are required for dipeptides. In view of the specificity requirements of CPase toward tripeptide analogs,[4] it was expected that only those unsubstituted dipeptides with aromatic amino acid or leucine residues in the C-terminal position would be hydrolyzed at appreciable rates. The data in Table I confirm this expectation. The hydrolytic rates of the glycyl dipeptides were significantly lower than those of the leucyl dipeptides. The fact that the tripeptide L-Leu-Gly-Gly is not hydrolyzed, under the conditions indicated in Table I, is an indication of the importance of the nature of the C-terminal residue. This enzyme preparation, unlike less purified preparations, did not split L-Leu-Gly. The hydrolysis of L-Arg-L-Tyr and L-Ser-L-Tyr has been reported by White and Landmann.[20]

The first-order "proteolytic coefficients" of substrates of CPase at an arbitrary concentration of 0.05 *M* have been generally used to express the hydrolytic susceptibilities of the compounds.[4] Just as in the case of tripeptide analogs,[13] the proteolytic coefficient for D-Leu-L-Tyr varied with the concentration of the substrate (Fig. 1). Therefore, the proteolytic coefficients of dipeptides as well as those of N-acyl dipeptides at arbitrary concentrations should be considered only relative measures of susceptibility to hydrolysis.

TABLE I

HYDROLYSIS OF PEPTIDES BY CARBOXYPEPTIDASE[a]

Peptide	Enzyme concn.[b]	Period of incubation (hr.)	Hydrolysis (%)
L-Leu-L-Tyr	25	1/2	19
D-Leu-L-Tyr	25	1/2	15
Gly-L-Try	50	20	13
Gly-L-Tyr	50	20	26
Gly-L-Leu	50	20	20
L-Leu-Gly-Gly	50	20	0
L-Leu-Gly	100	48	0
Cbz-Gly-L-Phe	0.2	1/2	15

[a] The reactions were carried out at *p*H 7.5. The substrate concentration was 0.02 *M*. [b] Micrograms protein nitrogen per ml.

The hydrolytic rates of a dipeptide and its N-acyl derivative at an arbitrary concentration cannot be compared quantitatively for another reason. The *p*H optimum for the hydrolysis of a limiting concentration of a dipeptide is higher than that of an N-acyl dipeptide, because the concentration of the substrate species of a dipeptide depends on the *p*H.[9]

Known inhibitors of CPase[21] such as 10^{-2} *M* pyrophosphate, 10^{-4} *M* CuCl and 10^{-4} *M* $HgCl_2$, affected the hydrolytic rates of Cbz-Gly-L-Phe and D-Leu-L-Tyr to the same degree. Furthermore,

(20) W. F. White and W. A. Landmann, THIS JOURNAL, **76**, 4193 (1954).

(21) E. L. Smith and H. T. Hanson, *J. Biol. Chem.*, **179**, 803 (1949).

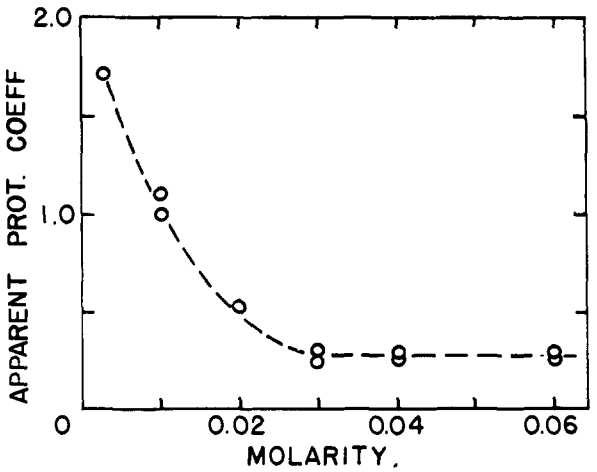

Fig. 1.—Dependence of the apparent proteolytic coefficient[4] on the concentration of D-Leu-L-Tyr.

the ratios of these rates were nearly constant for different enzyme preparations and in partially inactivated preparations.[22] These experiments constitute indirect evidence that CPase is responsible for the hydrolysis of the dipeptides reported in Table I; however, more direct evidence is presented in the following paper.[9]

N-Acyl Derivatives of L-Tyrosine.—The observation that the diastereoisomers D-Leu-L-Tyr and L-Leu-L-Tyr were hydrolyzed by CPase at the same rate[1,23] and that these rates were much greater than the hydrolytic rate of Gly-L-Tyr was the basis for detailed studies on derivatives of tyrosine. The data in Table II show that the nature of the

TABLE II

THE HYDROLYSIS OF N-ACYL DERIVATIVES OF L-TYROSINE BY CARBOXYPEPTIDASE

N-Acyl group	Concn., *M*	Relative rates[a] *p*H 7.5	*p*H 9.0
Glycyl	0.01	1	1
L-Seryl	.01	2	3
Acetyl	.02[b]	30	18
D-Leucyl	.01	140	120
L-Leycyl	.01	140	130
Acetyl-L-leucyl	0.01	12,000	

[a] Calculated from first-order velocity constants. An arbitrary value of one was assigned to the values for Gly-L-Tyr at each of the two *p*H's. The maximum reaction period (used for the glycyl and seryl derivatives) was two hours. [b] A higher concentration of acetyl-L-tyrosine was used, since the affinity of CPase for this compound is of a lower magnitude.[9]

acyl group has considerable influence on the hydrolytic susceptibility of a compound. These results cannot be explained simply on the basis of the acid strength of the acyl group which contributes the carbonyl group of the susceptible bond as suggested by Smith,[24] or on the basis of other known specificity requirements. In contrast to the very low hydrolytic rates of Gly-L-Tyr and L-Ser-L-Tyr, the hydrolytic rates of their carbobenzyloxy deriva-

(22) Unpublished results.

(23) Prior to the isolation of crystalline Cpase, E. Abderhalden and A. Bahn, *Fermentforsch.*, **11**, 399 (1930), reported the hydrolysis of D-Leu-L-Tyr and L-Leu-L-Tyr by "trypsin kinase." This hydrolysis was probably caused by CPase in the preparation.

(24) E. L. Smith, *Proc. Natl. Acad. Sci.*, **35**, 80 (1949).

tives are approximately equal to those of Cbz-L-Leu-L-Tyr and the reference substrate, Cbz-Gly-L-Phe.[22] This comparison demonstrates the effect of acylation of a dipeptide.

Stereospecificity of Carboxypeptidase.—The hydrolysis of D-Leu-L-Tyr and L-Leu-L-Tyr at equal rates indicates that the hydrolytic susceptibility of an unsubstituted dipeptide is not affected by the optical configuration of the α-carbon atom of the acyl residue. Acylation of these diastereoisomers, as shown in Table III, results in very striking changes in hydrolytic rates.

TABLE III

THE HYDROLYSIS OF N-ACYL DERIVATIVES OF D- AND L-LEUCYL-L-TYROSINE BY CARBOXYPEPTIDASE

N-Acyl group	Rate[a,b] L-Leu-L-Tyr	D-Leu-L-Tyr
None	150	140
Carbobenzyloxy-	29,000	5
Acetyl-	9,200	3
Chloroacetyl-	12,000	2
Glycyl-	5,700	..

[a] Apparent proteolytic coefficient $\times 10^3$. [b] The enzymatic reaction mixtures (pH 7.5) were 0.01 M in substrate. The enzyme concentrations, expressed as micrograms protein nitrogen per ml., were 1, 20 and 50, respectively, for the hydrolysis of the N-acyl derivatives of L-Leu-L-Tyr, the unsubstituted dipeptides, and the N-acyl derivatives of D-Leu-L-Tyr. The maximum reaction period (used for the N-acyl derivatives of D-Leu-L-Tyr) was 2 hr.

The N-acyl derivatives of L-Leu-L-Tyr are hydrolyzed approximately 40–200 times more rapidly than the unsubstituted dipeptide. The data are in agreement with the conclusion of Snoke and Neurath[5] that the presence of a secondary peptide bond is required for optimal activity. The differences between the hydrolytic rates of various N-acyl derivatives of L-Leu-L-Tyr are relatively small, indicating that the hydrolytic rates of these substrates are almost entirely determined by the nature of the amino acid residues which form the susceptible peptide bond and the presence or absence of the secondary peptide bond.

The N-acyl derivatives of D-Leu-L-Tyr are split several thousand times more slowly than the corresponding derivatives of L-Leu-L-Tyr. If steric hindrance were the only factor, the possibility would exist that derivatives of D-Leu-L-Tyr with small N-acyl substituents might be split at significantly greater rates than the carbobenzyloxy derivative. Even an acetyl group, however, is large enough to render the compound resistant to activation. The hydrolytic rates though very low are nonetheless significant, since acetyl-D-Leu-L-Tyr, for example, is split to the extent of 30% in 24 hr. Fu, *et al.*,[25] have reported significant hydrolysis of Cbz-D-Ala-L-Phe. The fact that Hanson and Smith detected no hydrolysis of Cbz-D-Try-Gly under conditions where Cbz-L-Try-Gly was slowly split[26] may be attributed to the fact that peptides containing glycine in the C-terminal position are poor substrates of CPase.

(25) S. J. Fu, S. M. Birnbaum and J. P. Greenstein, THIS JOURNAL, **76**, 6054 (1954).

(26) H. T. Hanson and E. L. Smith, *J. Biol. Chem.*, **179**, 815 (1949).

Aminopeptidase-like Contaminant.—Two of the three CPase preparations listed in Table IV were found capable of hydrolyzing L-Leu-Gly slowly, the hydrolysis occurring more rapidly at pH 8.0 than at pH 7.4. Enzymes A and B correspond to the six-times and four-times recrystallized preparations described earlier. The presence of the hydrolytic products was confirmed by paper chromatography. Since the absence of DL-Leu-Gly splitting activity has been used as one of the criteria for purifying CPase,[10] it was necessary to differentiate the hydrolysis of L-Leu-Gly from that of other dipeptides and tripeptide analogs.

TABLE IV.

EVIDENCE FOR AN AMINOPEPTIDASE-LIKE CONTAMINANT IN CARBOXYPEPTIDASE PREPARATIONS

Substrate	Hydrolysis,[a] % Enzyme A Control	Enzyme A DFP[b]	Enzyme B Control	Enzyme B DFP	Enzyme C Control	Enzyme C DFP
Cbz-Gly-L-Phe[c]	50	48	40			
D-Leu-L-Tyr[c]	25	22	19		44	
L-Leu-Gly[d]	0	0	12	0	74	11
D-Leu-Gly[d]			0	0	0	0

[a] The relative concentrations of each enzyme used were 1, 100, 300 and 300, respectively, for compounds in the order in which they are listed. [b] The DFP treatment consisted of pre-incubation of the enzymes with 10^{-3} M DFP for 20 min. (pH 7.5, 37°). [c] Reaction period was 30 min.; pH 7.5. [d] Reaction period was 48 hr.; pH 8.0.

Threefold evidence that the splitting of L-Leu-Gly is the result of a contaminant in CPase preparations is presented in Table IV: (a) L-Leu-Gly was not hydrolyzed by high concentrations of the six-times recrystallized preparation which was used in the foregoing studies; (b) diisopropylfluorophosphate (DFP) inhibited the splitting of L-Leu-Gly but had little effect on the hydrolysis of Cbz-Gly-L-Phe and D-Leu-L-Tyr; and (c) "preparation C" was capable of hydrolyzing D-Leu-L-Tyr and L-Leu-Gly but did not split D-Leu-Gly. This fact indicated the presence of two enzymes in "Enzyme C" which differ with respect to optical specificity toward the N-terminal amino acid and implies that the enzyme which splits L-Leu-Gly is an aminopeptidase-like enzyme.

Discussion

The specificity requirements of CPase toward dipeptides and N-acyl dipeptides[4] are similar in that substrates containing aromatic amino acid and leucine residues in the C-terminal position are preferentially split. The tripeptide L-Leu-Gly-Gly was not hydrolyzed under conditions adequate for the hydrolysis of dipeptides of aromatic amino acids. Thus, the nature of the C-terminal residue is of greater importance than the chain length of the peptide, even though CPase is considered a polypeptidase. The hydrolytic susceptibility of a dipeptide is also greatly influenced by the nature of the amino acid which contributes the carbonyl group of the peptide bond, as evidenced by the fact that the hydrolytic rates of the N-acyl derivatives of L-tyrosine vary more than a hundred-fold.

Except for D-amino acids, the effect of the N-acyl moiety of a dipeptide on hydrolytic susceptibility is no longer evident after the dipeptide has been

acylated. This is due, in part, to the fact that the influence of the secondary peptide bond on hydrolytic susceptibility is generally much greater than that of the acyl moiety of a dipeptide. Another factor is that a maximal activation rate for CPase substrates is evidently approached when the substrate is a tripeptide analog containing an aromatic amino acid residue in the C-terminal position.[4] As a consequence of these factors, the hydrolytic rates of dipeptides vary from a hundredth to a ten-thousandth as large as those of their corresponding N-acyl derivatives. The nature of an N-terminal acyl moiety separated from the susceptible bond by another amino acid residue, as in tripeptide analogs (refer to Table III), has relatively little effect on the hydrolytic susceptibility of the compound.

The presence of a secondary peptide bond not only enhances the hydrolytic susceptibility of a peptide[5] but is also responsible for the optical specificity of the enzyme for the second residue. The fact that D-Leu-L-Tyr and L-Leu-L-Tyr are split at nearly equal rates implies a lack of optical specificity for the acyl moiety of a dipeptide. However, in a tripeptide the second residue must be of the L-configuration[1,25,26] for optimal activity. The data on tripeptide analogs in which the second residues are of the D-configuration indicate that smaller side chains on the α-carbon atom of the D-amino acid residue render the compound somewhat more susceptible to activation.[27] Early evidence[28] indicated that a D-amino acid residue had no appreciable effect when it was the N-terminal residue of a tripeptide. Since compounds with large substituents on the α-carbon atom of the second residue, *e.g.*, Cbz-L-Try-L-Tyr, are excellent substrates[29] the resistance of acetyl-D-Leu-L-Tyr to CPase cannot be due primarily to the size of the substituents. It is, therefore, likely that both of the first two amino acid residues at the C-terminus of a polypeptide must be oriented stereospecifically on the enzyme surface in order to be activated at an optimal rate. Hydrogen bonding of the secondary peptide group has been proposed.[5]

A comparison of the action of exopeptidases on peptides containing D-amino acids reveals clear-cut differences in optical specificity. The optical specificity of exopeptidases toward dipeptides may be summarized as follows: an L-amino acid is an absolute requirement in one of the two positions, at the C-terminal position in the case of carboxypeptidases, and in the N-terminal position in the case of aminopeptidases. The degree of optical specificity for the other residue of the dipeptide can vary considerably within each class of enzymes.

The optical specificity of the aminopeptidase from the particulate fraction of kidney[30] is complementary to that of CPase (compare with data in Table III) in the following respects: an L-amino acid is required at the N-terminus of a peptide; diastereoisomers such as L-Ala-D-Ala and L-Ala-L-Ala are split at nearly equal rates; tripeptides in which the C-terminal residue is of the D-configuration are readily hydrolyzed; and tripeptides in which the second residue is of the D-configuration are not split. The optical specificity of amino*tri*peptidase[31] is somewhat similar to that of this kidney aminopeptidase toward tripeptides. On the other hand, the leucine aminopeptidases from intestine[32] and kidney[33] split L-Leu-L-Leu much more rapidly than its diastereoisomer L-Leu-D-Leu.

Carnosinase[34] hydrolyzes carnosine and the diastereoisomeric pair D-Ala-L-His and L-Ala-L-His at nearly equal rates but does not split D-carnosine (β-Ala-D-His). The primary specificity of this enzyme is directed toward the L-histidine residue. From a consideration of the optical specificities of carboxypeptidases[4,25] and aminopeptidases[35] it is more likely that carnosinase is a carboxypeptidase rather than an aminopeptidase as suggested by Hanson and Smith.[34]

Kidney acylase,[25] which is a carboxy*di*peptidase, hydrolyzes L-alanyl dipeptides several hundred times more rapidly than the corresponding D-alanyl dipeptides. The differences between the hydrolytic rates of the D- and L-α-chloropropionyl derivatives of L-amino acids are less pronounced.

The use of dipeptides containing D-amino acids as specific substrates provides a unique approach to the problem of identifying and isolating exopeptidases from crude tissue preparations in the presence of other exopeptidases. Robinson, *et al.*,[30] have used Gly-D-Ala as a specific substrate to isolate an aminopeptidase from kidney. In this paper D-Leu-L-Tyr, D-Leu-Gly and their optical isomers were used to show differences between the optical specificities of CPase and the leucylglycine-splitting contaminant in certain preparations. These examples show that peptides containing D-amino acids may be used successfully in distinguishing between exopeptidases.

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(27) A correlation of the data in Table III and those of Fu, *et al.*,[28] indicates that the hydrolytic rate of Cbz-D-Ala-L-Phe is about tenfold greater than that of Cbz-D-Leu-L-Tyr. Since phenylalanine and tyrosine are nearly equivalent,[4] the differences in hydrolytic rates may be attributed to the fact that alanine has a smaller side chain than leucine.

(28) E. Waldschmidt-Leitz and A. K. Balls, *Ber.*, **63**, 1203 (1930).

(29) E. L. Smith, *J. Biol. Chem.*, **175**, 39 (1948).

(30) D. S. Robinson, S. M. Birnbaum and J. P. Greenstein, *ibid.*, **202**, 1 (1953).

(31) J. S. Fruton, V. A. Smith and P. S. Driscoll, *ibid.*, **173**, 457 (1948).

(32) E. L. Smith and W. J. Polglase, *ibid.*, **180**, 1209 (1949).

(33) E. L. Smith and D. H. Spackman, *ibid.*, **212**, 271 (1955).

(34) H. T. Hanson and E. L. Smith, *ibid.*, **179**, 789 (1949).

(35) E. L. Smith, *Advances in Enzymol.*, **12**, 191 (1951).