ing radiations on the constitutive amino acids of DNase I with that on amino acid solutions seems unwarranted since the experimental conditions are not comparable. The ionic yield in terms of ammonia formation is a function of amino acid concentration, pH and radiation intensity and, in this respect, there seems no real basis for comparison. Furthermore, the respective state in which the amino acids are irradiated are obviously quite different.

SUMMARY

1. Significant changes in the amino acid composition of DNase I were observed after exposure of the enzyme to irradiation. Radiation-induced deamination appears to be the most important factor in the production of this change.

2. Exposure to large doses of ionizing radiation resulted in a significant change in the ultraviolet spectrum of DNase I. Although the absorption at 250 mμ increased linearly with radiation dose, the enzyme activity decreased exponentially.

3. These studies indicate that large doses of ionizing radiations sufficient to destroy the enzymic properties of this protein did not bring about an equally extensive change in the amino acid composition.

REFERENCES

1 E. S. G. BARRON, J. AMBROSE AND P. JOHNSON, *Radiation Research*, 2 (1955) 145.
2 S. OKADA, *Arch. Biochem. Biophys.*, (in the press).
3 G. L. FLETCHER AND S. OKADA, *Nature*, 176 (1955) 882.
4 G. GEHRMANN AND S. OKADA, *Biochim. Biophys. Acta*, 23 (1957) 621.
5 O. D. KOWLESSAR, K. I. ALTMAN AND L. H. HEMPELMANN, *Arch. Biochem. Biophys.*, 52 (1954) 362.
6 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 893.
7 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 907.
8 J. R. SPIES AND D. C. CHAMBERS, *Anal. Chem.*, 21 (1949) 1249.
9 E. S. G. BARRON AND P. FINKELSTEIN, *Arch. Biochem. Biophys.*, 41 (1952) 212.
10 W. M. DALE AND C. RUSSELL, *Biochem. J.*, 62 (1956) 50.
11 G. STEIN AND J. WEISS, *J. Chem. Soc.*, (1949) 3256.

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THE ACTION OF CHYMOTRYPSIN ON N-ALKYL DERIVATIVES OF PHENYLALANINE ETHYL ESTER

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Studies by NEURATH *et al.*[1] have shown that chymotrypsin can act on ester linkages as well as on amide bonds, provided that the other specificity requirements are met. Thus, chymotrypsin readily hydrolyzes the ester group of such compounds as benzoyl L-tyrosine ethyl ester, benzoyl L-phenylalanine ethyl ester, acetyl L-tyrosine ethyl ester, etc. It was first found that the replacement of the "secondary peptide bond", such as the benzoyl amino or the acetyl amino group of the above compounds, by a

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free amino group greatly decreases the susceptibility of the resulting compound to chymotryptic action. This and other observations were explained by the assumption that the higher susceptibility of compounds bearing a "secondary peptide bond" is the result of the formation of two hydrogen bridges between this peptide bond and a complementary peptide bond on the enzyme surface. However, it was found by GOLDENBERG AND GOLDENBERG[2] that L-phenylalanine ethyl ester was readily hydrolyzed by chymotrypsin if the cleavage was carried out at pH 6.4 instead of the slightly alkaline medium (pH 7.8) usually employed in experiments with chymotrypsin. In view of these findings, it appeared of interest to study the action of chymotrypsin on those derivatives of phenylalanine ethyl ester in which one hydrogen of the amino group was replaced by an alkyl group.

EXPERIMENTAL

We prepared the following compounds: DL-N-ethylphenylalanine ethyl ester, DL-N-methylphenylalanine ethyl ester, and L- and D-N-methylphenylalanine ethyl ester.

L-N-Methylphenylalanine was prepared from D-phenylalanine according to the method of IZUMIYA *et al.*[6,7]. $[\alpha]_D^{23}$ + 49.5° (in 0.15 *N* NaOH). FISCHER AND LIPSCHITZ[8] have reported $[\alpha]_D^{18}$ + 49.7°. The substance was converted to the ethyl ester hydrochloride by the method used by FISCHER in the preparation of L-tyrosine ethyl ester[9]. The product obtained was recrystallized from alcohol-ether. M.p. 133–134°C (Fischer-Johns melting point apparatus). Found: N, 5.7; neutral equivalent, 248 (Willstätter-Waldschmidt-Leitz titration), 247 (Sørensen formol titration). Calculated: N, 5.7; neutral equivalent, 244. $[\alpha]_D^{20}$ + 9.4° (5% in water). IZUMIYA AND FRUTON[7] have reported m.p. 133°C and $[\alpha]_D^{22}$ + 7.8° (2% in water) for this compound.

D-N-Methylphenylalanine ethyl ester hydrochloride was prepared from L-phenylalanine in the same manner as the L-isomer. M.p. 133–134°C. Found: N, 5.8; neutral equivalent, 239 (Sørensen formol titration). Calculated: N, 5.7; neutral equivalent, 244. $[\alpha]_D^{22}$ — 9.6° (5% in water).

DL-N-Methylphenylalanine ethyl ester hydrochloride was prepared from DL-phenylalanine in a way similar to the L- and D-compounds. Found: N, 5.8; neutral equivalent, 240 (Sørensen formol titration). Calculated: N, 5.7; neutral equivalent, 244.

DL-N-Ethylphenylalanine ethyl ester hydrochloride was prepared from DL-α-bromo-β-phenylpropionic acid[10] by treating it with ethylamine and by esterification of the obtained product by FISCHER's method. The substance was recrystallized from ethanol. Found: N, 5.3; neutral equivalent, 257 (Willstätter-Waldschmidt-Leitz titration). Calculated: N, 5.4; neutral equivalent, 258.

DL-Phenylalanine ethyl ester hydrochloride was prepared from DL-phenylalanine by FISCHER's method. The substance was recrystallized from ethyl acetate. Found: neutral equivalent, 228 (Willstätter-Waldschmidt-Leitz titration). Calculated: 230.

L-Phenylalanine ethyl ester hydrochloride was prepared from L-phenylalanine in a similar way to the DL-compound. It was recrystallized from alcohol-ether. Found: neutral equivalent, 228 (Willstätter-Waldschmidt-Leitz titration). Calculated: 230.

Worthington crystalline, salt-free chymotrypsin was used. As can be seen from the results summarized in Table I, at a pH range varying between 5.7 and 7.7 none of the N-alkyl compounds mentioned was susceptible to the action of chymotrypsin.

DISCUSSION

Considering the fact that N-benzoyl and N-acetyl derivatives of phenylalanine ethyl ester, as well as phenylalanine ethyl ester itself, are readily attacked by chymotrypsin, the resistance of N-methyl and N-ethyl derivatives of phenylalanine ethyl ester to chymotryptic activity is somewhat surprising. This resistance could be explained by a diminished tendency of the alkyl-substituted amino group to form a hydrogen bond, by proton donation, with an appropriate group on the enzyme surface. The following

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TABLE I

THE ACTION OF CHYMOTRYPSIN ON PHENYLALANINE ETHYL ESTER AND ITS N-ALKYL DERIVATIVES

Substrate	*pH*	*5.7*			*6.6*			*6.9*			*7.7*		
	Time, minutes	*0*	*10*	*20*	*0*	*10*	*20*	*0*	*10*	*20*	*0*	*10*	*20*
DL-PEE*	Colorimeter reading**	465	400	365	470	355	296	465	300	244	465	350	294
DL-PEE***	Colorimeter reading**	470	460	450	465	460	450	470	465	465	465	460	455
DL-N-CH_3-PEE*	Colorimeter reading**	440	435	430	435	420	415	450	454	450	475	480	475
DL-N-CH_3-PEE***	Colorimeter reading**	435	430	425	435	420	420	445	450	450	465	475	475
DL-N-C_2H_5-PEE*	Colorimeter reading**	445	445	440	460	450	455	460	445	435	470	475	475
DL-N-C_2H_5-PEE***	Colorimeter reading**	435	435	435	455	450	455	465	450	440	475	475	470
L-PEE*	Colorimeter reading**	236	168	138	220	80	30	228	60	13	236	120	32
L-PEE***	Colorimeter reading**	234	234	232	214	216	214	230	228	228	222	220	222
L-N-CH_3-PEE*	Colorimeter reading**	218	218	220	224	216	216	228	228	222	222	220	222
L-N-CH_3-PEE***	Colorimeter reading**	214	218	218	222	218	216	230	228	228	222	220	222
D-N-CH_3-PEE*	Colorimeter reading**	220	220	216	224	216	212	228	228	222	216	212	216
D-N-CH_3-PEE***	Colorimeter reading**	215	212	218	220	216	214	226	224	216	218	218	218

* The abbreviation PEE is used for phenylalanine ethyl ester hydrochloride.
** Klett-Summerson colorimeter, filter 54.
*** No enzyme added.

Each reaction mixture contained: Phosphate buffer, 0.1 *M*, 6 ml; chymotrypsin, 0.4 mg; substrate, approximately 60 μmoles (L-substrates) or 120 μmoles (DL-substrates). Temperature, 30°C. 1 ml samples were tested by HESTRIN's method (10 min treatment with hydroxylamine prior to the addition of HCl and $FeCl_3$).

consideration may serve as an alternative explanation of our findings. GOLDENBERG AND GOLDENBERG[2] found that optimal hydrolysis of phenylalanine ethyl ester occurred at pH 6.4 when the rate of cleavage was measured by titration of the liberated acid. However, when they investigated the action of chymotrypsin on the same compound by measuring the disappearance of the ester, using HESTRIN's hydroxamic acid method[3], they found an apparent increase in the rate of the reaction and a distinct broadening of the pH range of enzymic activity with a shift towards the alkaline region. This discrepancy can easily be understood if one realizes that HESTRIN's method measures, in addition to hydrolysis, the disappearance of ester caused by transpeptidation reactions whose pH optimum may differ from that of the hydrolysis[4]. Since we have not found any activity of chymotrypsin with our compounds using HESTRIN's method, it appears that neither hydrolysis nor replacement reactions occurred. In order to explain the lack of susceptibility of the N-alkyl derivatives to chymotryptic activity we suggest that phenylalanine ethyl ester is not hydrolyzed directly by chymotrypsin but is first converted, by a transpeptidation reaction, to phenylalanylphenylalanine ethyl ester (or to the ester of a higher peptide). This compound bearing a "secondary peptide bond" is then rapidly hydrolyzed by the enzyme. The inability of chymotrypsin to carry out transpeptidation reactions with the N-alkyl-substituted esters would then account for the resistance of such compounds to the action of the enzyme. It may be recalled that TAUBER[5] obtained phenylalanylphenylalanine ethyl ester on treating phenylalanine ethyl ester with chymotrypsin at pH 8.8. He also found that chymotrypsin did not markedly hydrolyze this dipeptide ester at pH 7.7. If our assumption is correct, namely that this ester acts as an intermediary compound in the chymotryptic hydrolysis of phenylalanine ethyl ester, it would seem that the optimal pH for its cleavage must be in the slightly acid region.

Experiments to check the validity of the above explanations are under way.

SUMMARY

The action of chymotrypsin on N-methyl and N-ethyl derivatives of phenylalanine ethyl ester at a pH range varying between 5.7 and 7.7 was investigated. Neither hydrolysis nor replacement reactions could be detected.

REFERENCES

[1] H. NEURATH AND G. W. SCHWERT, *Chem. Revs.*, 46 (1950) 69.
[2] H. GOLDENBERG AND V. GOLDENBERG, *Arch. Biochem.*, 29 (1950) 154.
[3] S. HESTRIN, *J. Biol. Chem.*, 180 (1949) 249.
[4] M. GREEN AND H. NEURATH, *The Proteins*, Vol. II, Part B, Academic Press Inc., New York, 1954, p. 1137.
[5] H. TAUBER, *J. Am. Chem. Soc.*, 74 (1952) 847.
[6] N. IZUMIYA, A. NAGAMATSU AND S. ÔTA, *Kyushu Mem. Med. Sci.*, 4 (1953) 1.
[7] N. IZUMIYA AND J. S. FRUTON, *J. Biol. Chem.*, 218 (1956) 59.
[8] E. FISCHER AND W. LIPSCHITZ, *Ber.*, 48 (1915) 360.
[9] E. FISCHER, *Ber.*, 34 (1901) 433.
[10] C. S. MARVEL, *Org. Syntheses*, 21 (1941) 99.

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