6-Uracilsulfonamide (VII).—The dimethoxysulfonamide (above) was demethylated by autoclaving a suspension of 5.5 g. in 750 ml. of 0.1 *N* hydrochloric acid for two hours at 250°F. (15 p.s.i.). The solution was brought to dryness under reduced pressure and the residue was recrystallized from water. The fine crystals of 6-uracilsulfonamide gradually decomposed above 235°; the yield (including work-up from mother liquor) was 2.8 g. (58%). The analytical sample was recrystallized twice more from the same solvent. The absorption is given in Table I.

Anal. Calcd. for $C_4H_5N_3O_4S$: C, 25.13; H, 2.64; N, 21.98; S, 16.77. Found: C, 25.20; H, 2.67; N, 21.86; S, 16.57.

2,4-Dimethoxy-6-methylmercaptopyrimidine (VIII).—A solution of 6.88 g. (0.04 mole) of 2,4-dimethoxy-6-pyrimidinethiol[2] in 80 ml. of 2.5% sodium hydroxide was treated dropwise with 7.2 g. (0.056 mole) of dimethyl sulfate. The mixture was stirred for 1.5 hours with the occasional addition of enough 10% sodium hydroxide to keep the mixture alkaline. The insoluble methylmercapto derivative crystallized when the reaction mixture was cooled in ice, and was apparently reasonably pure after it was collected and washed with water; yield 5.9 g. (80%), m.p. 44–46°. Recrystallization was effected by dissolving the compound in hot ethanol (95%) and then adding an equal volume of water to the cooled solution. This returned 5 g., m.p. 45–46°. The analytical sample was recrystallized an additional time.

Anal. Calcd. for $C_7H_{10}N_2O_2S$: S, 17.22. Found: S, 17.31.

2,4-Dimethoxy-6-pyrimidine Methyl Sulfone (IX).—A solution of 5 g. of the above methylmercapto derivative in 135 ml. of 88% formic acid was treated with 13.5 ml. of Superoxol and allowed to stand for 3 hours. It was then diluted with an equal volume of water and allowed to stand overnight. The residue obtained by evaporating the solution to dryness under reduced pressure was collected by means of a small amount of cold absolute ethanol and sucked dry. A recrystallization from absolute ethanol afforded the sulfone in the form of long needles; yield 4.81 g. (82%), m.p. 122–123°. The analytical sample was recrystallized a second time.

Anal. Calcd. for $C_7H_{10}N_2O_4S$: C, 38.52; H, 4.62; N, 12.84; S, 14.69. Found: C, 38.48; H, 4.80; N, 12.87; S, 14.60.

6-Uracil Methyl Sulfone (X).—The dimethoxy sulfone (above) was demethylated by autoclaving a suspension of 4.8 g. in 700 ml. of 0.1 *N* hydrochloric acid for two hours at 250°F. (15 p.s.i.). The solution was brought to a small volume under reduced pressure and the crystalline material which separated out was collected (3 g.) and recrystallized from water. By reworking the mother liquor a total of 1.9 g. (45%) of the recrystallized product was obtained, m.p. 299–300° dec. The analytical sample was recrystallized from glacial acetic acid and washed with petroleum ether, m.p. 307–308°. The absorption data are found in Table I.

Anal. Calcd. for $C_5H_6N_2O_4S$: C, 31.58; H, 3.18; N, 14.73; S, 16.86. Found: C, 31.73; H, 3.37; N, 14.44; S, 16.70.

In later work it was found more advantageous to demethylate the demethoxy sulfone by heating it with a 90% acetic acid solution which was 0.2 *N* with respect to hydrochloric acid. This procedure afforded a somewhat higher yield and gave a product which was chromatographically homogeneous after only a single recrystallization. Thus a mixture of 21.8 g. of the demethoxy sulfone (IX), 1575 ml. of glacial acetic acid and 175 ml. of 2 *N* hydrochloric acid was heated under reflux for three hours. The solution was evaporated to dryness under reduced pressure and the residue was recrystallized from water. The uracil methyl sulfone was obtained in the form of fine rods; yield 10.1 g. (54%), m.p. 307–308° dec.

New Haven, Conn.

[Contribution from the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health]

Influence of Optically Active Acyl Groups on the Enzymatic Hydrolysis of N-Acylated-L-amino Acids

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L- and D-alanine were converted to the corresponding optically active α-chloropropionic acids without loss of configuration, and the halogenated acids combined through their acid chlorides with glycine, L-alanine, L-butyrine and L-norvaline for studies with renal acylase I, and with L-phenylalanine and L-tyrosine for studies with pancreatic carboxypeptidase. The acylated amino acids with L-acyl substituents were hydrolyzed by renal acylase at greater rates than those with D-acyl substituents, but the difference between the rates for the L- and D-acyl substituents was very much greater when the acyl groups were alanyl than when they were α-chloropropionyl. With increasing length of the side-chain of the terminal L-amino acid this difference increased in the case of the α-chloropropionyl derivatives and was practically constant in the case of the alanyl derivatives. In the case of pancreatic carboxypeptidase, the difference in rates between L- and D-acyl derivatives was relatively small except for the carbobenzoxyalanyl derivatives. Unlike renal acylase I, pancreatic carboxypeptidase attacks the L-chloropropionyl residue much more readily than it does the L-alanyl or propionyl.

Acylase I is a soluble, intracellular carboxypeptidase which catalyzes the hydrolysis of a wide variety of N-acetylated amino acids at rates which are dependent upon the nature of the N-acyl group and of the terminal amino acid residue.[1-8] N-Chloroacetyl-L-amino acids are hydrolyzed by acylase I at faster rates than are corresponding N-glycyl-L-amino acids.[3] On the other hand, N-DL-chloropropionyl-L-alanine is hydrolyzed at a much slower rate than is either L-alanyl-L-alanine,[3] or N-propionyl-L-alanine,[3] and it would appear that electronic effects alone are insufficient to account for the influence of the nature of substituents in the N-acyl group on the susceptibility of the substrate to acylase I. Moreover, although L-alanyl amino acids are hydrolyzed much more rapidly than are D-alanyl amino acids, inspection of the hydrolytic curve of DL-chloropropionyl-L-alanine[2] suggests that the susceptibility of the two optical forms to acylase I is not greatly different. Yet one of these two forms corresponds in optical configuration to that of L-alanyl-L-alanine, and the other to that of D-alanyl-L-alanine.

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

(2) P. J. Fodor, V. E. Price and J. P. Greenstein, *ibid.*, **182**, 467 (1950).

(3) K. R. Rao, S. M. Birnbaum, R. B. Kingsley and J. P. Greenstein, *ibid.*, **198**, 507 (1952).

(4) K. R. Rao, S. M. Birnbaum and J. P. Greenstein, *ibid.*, **203**, 1 (1953).

(5) W. S. Fones and M. Lee, *ibid.*, **201**, 847 (1953).

(6) S-C. J. Fu and S. M. Birnbaum, This Journal, **75**, 918 (1953).

(7) W. S. Fones and M. Lee, *J. Biol. Chem.*, in press.

(8) J. P. Greenstein, S. M. Birnbaum and M. C. Otey, *ibid.*, **204**, 307 (1953).

To investigate these phenomena in greater detail, L- and D-alanine were converted to the corresponding optically active α-chloropropionic acids which were combined through their acid chloride derivatives with glycine, L-alanine, L-butyrine[9] and L-norvaline. The rates of hydrolysis of these derivatives by renal acylase I have been compared with those for the corresponding L- and D-alanyl derivatives of these amino acids. Inasmuch as the hydrolytic rates of homologous N-acylated straight-chain amino acids increase with increasing side-chain length to a maximum with N-acylated norvaline,[1,6,10] it was anticipated that the effects of the optically active acyl groups would be particularly revealed in such a series of compounds. For purposes of comparison with the action of another variety of acylase, data on the use of crystalline pancreatic carboxypeptidase with optically active and other N-acyl groups substituted in L-tyrosine and L-phenylalanine are included. It is assumed that when the optically active alanine isomers are converted to the α-chloropropionic acids the optical configurations are fully retained.[11-13] Table I summarizes the data obtained with renal acylase I.

TABLE I

SUSCEPTIBILITY OF ACYLATED L-AMINO ACIDS WITH OPTICALLY ACTIVE ACYL GROUPS TO RENAL ACYLASE I

Terminal amino acid residue	Rate of hydrolysis of[a] α-Chloropropionyl derivatives L form	D form	L:D	Alanyl derivatives L form	D form	L:D
Glycine	23	13	1.8	240	0.5	480
L-Alanine	87[b]	19	4.6	1200	3	400
L-Butyrine	290	28	10.4	5650	12	470
L-Norvaline	1240	106	11.7	9500	28	340

[a] In terms of micromoles substrate hydrolyzed at 37° per hour per mg. of N. Digests composed of 1 ml. of 0.1 *M* phosphate buffer at *p*H 7.0, 1 ml. of 0.025 *M* neutralized substrate and 1 ml. suitably diluted enzyme. [b] DL-Bromopropionyl-L-alanine is hydrolyzed under these conditions at a rate of less than 3 micromoles per hour per mg. of N.

It is not surprising or unexpected to note that the L-acylated amino acids are more susceptible than the corresponding D-acylated amino acids, or that all the acylated amino acids, whether the acyl radical is chloropropionyl or alanyl, or whether it is L- or D- in configuration, increase in susceptibility with increase in the length of the side-chain. What is particularly novel is the difference in the ratios of the rates for the L- and D-acylated amino acids, for whereas in the α-chloropropionyl series this ratio increases with increasing chain length of the terminal amino acid, that in the alanyl series is nearly independent of this factor. Furthermore, the magnitude of this ratio is very much greater in the latter series. Substitution of an amino group for the halogen greatly exaggerates the influence of the optical configuration of the N-acyl moiety. Again, for the same terminal amino acid residue, the L-alanyl derivative is much more susceptible than is the corresponding L-chloropropionyl derivative, whereas the D-alanyl derivative is less susceptible than is the corresponding D-chloropropionyl derivative.

Before an interpretation of these results may be ventured, it is necessary to know whether the substrates are being affected by a single enzyme in the acylase I preparation, or by several possible enzymatic components. Previous studies using chloroacetyl-L-alanine and glycyl-L-alanine strongly suggested that both substrates were hydrolyzed by a single enzyme. Table II in which data are collected on the partial and progressive denaturation of acylase I likewise indicates that the activity of acylase I toward the presently employed substrates is largely if not wholly due to a single enzymatic entity.

TABLE II

PROGRESSIVE ACID DENATURATION OF RENAL ACYLASE I[a]

Period of incubation, min.	% activity remaining toward L-Chloropropionyl-L-butyrine	L-Alanyl-L-butyrine
0	100	100
10	84	85
20	70	68
40	66	63
60	59	56
90	50	49
	L-Alanyl-L-butyrine	D-Alanyl-L-butyrine
0	100	100
30	67	66
60	50	52

[a] Acylase I powder was dissolved in a concentration of 6.25 mg. per ml. in 0.1 *M* acetate buffer at *p*H 4.7 and kept at 37°. At the indicated times 1 ml. was removed and added to 9 ml. of cold 0.1 *M* phosphate buffer at *p*H 7, and the digests treated with suitably diluted aliquots of this neutral solution of enzyme. The activity at each interval was determined in terms of rates as in Table I.

The susceptibility of a particular acylated amino acid is influenced by at least three factors: (a) the optical configuration of the terminal amino acid residue, (b) the nature of the carbon chain of the terminal amino acid residue and (c) the nature of the acyl radical. The influence of the nature of the acyl radical is in turn expressible in terms of three properties, namely, (a) electronic, (b) steric and (c) optical. The susceptibility of the substrate will therefore be the resultant of these several influences. Thus, the rate of hydrolysis of chloroacetyl amino acids is faster than that of corresponding acetyl amino acids, and the rate of hydrolysis of the monohalogen acetylalanines,[5] and of the monohalogen propionylalanines[3] decreases in the order of decreasing electronegativity of the halogen atom. However, L- or D-chloropropionylalanine is hydrolyzed at a much slower rate than is propionylalanine, and it must be assumed that steric factors are more important in this case than are electronic. Since electronic and steric factors would each be similar for L- and D-chloropropionylalanine, the influence responsible for the difference in susceptibility between these substrates must be primarily optical. Yet this optical influence is not as great as occurs when the substituent in the propionyl radical is an α-amino group (Table I), and

(9) L-Amino-*n*-butyric acid, *cf.* J. P. Greenstein, *Advances Prot. Chem.*, in press (1954).

(10) C. G. Baker and A. Meister, THIS JOURNAL, **73**, 1336 (1951).

(11) K. Freudenberg, W. Kuhn and I. Bumann, *Ber.*, **63**, 2380 (1930).

(12) W. A. Cowdrey, E. D. Hughes and C. K. Ingold, *J. Chem. Soc.*, 1243 (1937).

(13) A. Neuberger, *Advances Protein Chem.*, **4**, 297 (1948).

it would appear that the presence of this group in L-configuration would bring into play an additional polar point of attachment on the enzyme surface, and this reinforcement may be quite powerful in effecting the hydrolysis of the substrate. When the acyl radical is D in configuration this additional point on the enzyme surface may still be induced, but since the most advantageous orientation between enzyme and substrate could not conceivably occur, the result would be a prepared but ineffectual enzyme surface and a degree of susceptibility of the substrate lower even than that of the corresponding D-chloropropionyl derivative, or for that matter, of the corresponding glycyl derivative. This hypothetical "optical point of attachment" on the enzyme may therefore be conjectured to be optimally induced by an α-amino group in an acyl radical of L-configuration, and to a much lesser degree by halogen groups. Where the α-amino group is substituted in an optically symmetric radical as in acetyl, this "point" is not induced, and the highly electronegative chloroacetyl radical confers on the substrate a greater susceptibility to enzymatic hydrolysis than does the glycyl. The concept of an influence by the nature of the substrate on the enzyme which hydrolyzes it is implicit in any theory of enzyme–substrate interaction, and it may be assumed that any accommodation by the enzyme to achieve a more perfect orientation would bring the molecule into a configuration more closely approaching the activated state.[14]

A corresponding study on pancreatic carboxypeptidase reveals a somewhat different picture than that of renal acylase (Table III). The L-chloropropionyl as well as the L-alanyl derivatives of L-phenylalanine are more susceptible than are the corresponding D-acylated derivatives, but this difference in susceptibility is not at all striking. When, however, the alanyl derivatives are carbobenzoxylated as in carbobenzoxy-L-alanyl-L-phenylalanine and carbobenzoxy-D-alanyl-L-phenylalanine, a very considerable difference in susceptibility occurs, a finding compatible with earlier data by Hanson and Smith[15] and Yanari and Mitz[16] with other types of substrates. It has been suggested that the so-called secondary peptide bond in such substrates as carbobenzoxyglycyl-L-phenylalanine facilitates hydrolysis of the compound by carboxypeptidase.[14] That the terminal amino acid residue must be exclusively of the L-configuration for attack by carboxypeptidase is well established,[15,17] and it seems that the same optical specificity of the enzyme extends also to the second amino acid residue in such a substrate as well. From the limited data in Table III it would appear that in the absence of an acyl group, *i.e.*, when the α-amino group is free, the optical configuration of the second amino acid is of a much lesser degree of importance.

TABLE III

SUSCEPTIBILITY OF ACYLATED L-PHENYLALANINE WITH OPTICALLY ACTIVE AND OTHER ACYL GROUPS TO PANCREATIC CARBOXYPEPTIDASE ACCORDING TO FIRST-ORDER KINETICS

Derivative of L-phenylalanine	First-order velocity constant per mg. N per ml.[c]
L-Chloropropionyl[a]	0.264
D-Chloropropionyl[a]	.224
L-Alanyl[b]	.047
D-Alanyl[b]	.018
Carbobenzoxy-L-alanyl	71.0
Carbobenzoxy-D-alanyl	0.265
Acetyl	0.034
Chloroacetyl	4.13
Propionyl	0.105
Glycyl[b]	0.020

[a] Corresponding data for the derivatives of L-tyrosine are 0.304 and 0.214. [b] The hydrolysis of these compounds followed zero-order kinetics under the present conditions; the rates however have been approximated in terms of a first-order reaction for purposes of comparison with other data in the table. [c] Digests were composed of 1 ml. of 0.1 *M* veronal buffer at *p*H 7.45, 1 ml. of 0.025 *M* neutralized substrate, and 1 ml. of suitably diluted enzyme; incubation temperature 37°. The reaction constant is expressed as $C^{37}_{0.005}$, *cf.* ref. 14.

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

Experimental

L- and D-Chloropropionic Acids and their Acid Chlorides.—Optically pure L- and D-alanine, $[\alpha]^{25}D$ +14.4 and −14.4° (*c* 2, 5 *N* HCl), respectively, were obtained by resolution of acetyl-DL-alanine through the action of acylase I.[1] The amino acids were converted to the corresponding α-chloropropionic acids by a modification of the method of Fischer.[18] An amount of 17.8 g. (0.2 *M*) of either isomer was dissolved in 250 ml. of 6 *N* HCl and the solution brought to 0°. With vigorous stirring, 22 g. (0.32 *M*) of freshly pulverized sodium nitrite was added in small portions, and at such a rate that the temperature of the solution remained between 0 and 5°. About 2.5 hours were required for this purpose, and the stirring was continued at 0° for 4 hours longer. The solution was then extracted with ether, the ethereal extract dried over $CaCl_2$ for 10 hours, and the solvent removed by evaporation. The slightly yellow residual liquid was fractionally distilled twice, and for analysis the colorless liquid was further fractionated in a 45-cm. Podbielniak column[19] (50 calculated theoretical plates under atmospheric pressure). The take-off ratio was 1 to 5, and the boiling point was 77° at 10 mm. pressure. The highest yield obtained was 32% of the theoretical. The L- or D-chloropropionic acid showed no measurable racemization on standing at −10° in the dark for 4 weeks.[20]

Anal. Calcd. for $C_3H_5O_2Cl$: Cl, 32.6. Found (for L-chloropropionic acid from L-alanine): Cl, 32.5; $\alpha^{25}D$ −18.2° (*l* = 10 cm.), $[\alpha]^{25}D$ −14.6°, d^{25} 1.2485 and $n^{25}D$ 1.4322. Found (for D-chloropropionic acid from D-alanine): Cl, 32.4; $\alpha^{25}D$ +18.2° (*l* = 10 cm.), $[\alpha]^{25}D$ +14.6°, d^{25} 1.2485, and $n^{25}D$ 1.4321.

The corresponding acid chlorides were prepared by chilling the α-chloro acids to −10° and adding a slight excess of chilled thionyl chloride plus a few drops of phosphorus trichloride. The reaction mixture was shaken vigorously in a bath at −10° for some 15 minutes, and the resulting homogeneous liquid heated on the steam-bath for 4 hours

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

(16) S. S. Yanari and M. A. Mitz, *Federation Proc.*, **13**, 326 (1954).

(17) M. A. Stahmann, J. S. Fruton and M. Bergmann, *J. Biol. Chem.*, **164**, 753 (1946).

(18) E. Fischer, *Ber.*, **40**, 489 (1907).

(19) T. P. Carney, Laboratory Fractional Distillation, New York, 1949.

(20) Under the same conditions, using 6 *N* HBr instead of HCl, and without the use of the Podbielniak column, the corresponding L- and D-bromopropionic acids were prepared in yields of 60–65% of the theoretical. These compounds possessed, respectively, $\alpha^{25}D$ −46° and +46° (*l* = 10 cm.), $[\alpha]^{25}D$ −27.2° and +27.2°, d^{25} 1.6915 and 1.6930, $n^{25}D$ 1.4705 and 1.4722, and Br found 52.2 and 52.1 (calcd. Br 52.2). The liquids distilled at 78° under 4 mm. Further distillation through a Podbielniak column led to products with generally lower optical rotation values, and the instability of the α-bromopropionic acids was noted on standing even at −10° in the dark. The α-iodo- and α-fluoropropionic acids could not be prepared by the present procedure.

TABLE IV

PHYSICAL CONSTANTS OF L- AND D-CHLOROPROPIONYL-L-AMINO ACIDS

Compound	M.p.[a]	$[\alpha]^{24}D$ in:[b] H_2O	Methanol	Calcd. N	Calcd. Cl	Found N	Found Cl
L-Chloropropionylglycine	127	−21.4	−31.3	8.5	21.4	8.6	21.4
D-Chloropropionylglycine	127	+21.4	+31.2	8.5	21.4	8.2	21.5
L-Chloropropionyl-L-alanine	156[c]	−66.5	−47.6	7.8	19.8	8.0	19.6
D-Chloropropionyl-L-alanine	156[c]	−35.7	− 9.8	7.8	19.8	7.9	19.6
L-Chloropropionyl-L-butyrine	94[d]	−49.4	−31.7	7.2	18.3	7.1	18.4
D-Chloropropionyl-L-butyrine	94[d]	−26.4	− 3.4	7.2	18.3	7.4	18.3
L-Chloropropionyl-L-norvaline	48–50	−40.0	−25.4	6.8	17.1	6.7	17.0
D-Chloropropionyl-L-norvaline	85	−20.6	− 1.6	6.8	17.1	6.7	16.9
L-Chloropropionyl-L-phenylalanine	89	....	+41.5	5.5	13.9	5.6	13.7
D-Chloropropionyl-L-phenylalanine	112	....	+30.2	5.5	13.9	5.4	14.0
L-Chloropropionyl-L-tyrosine	155[e]	....	+51.1	5.2	13.1	5.3	12.7
D-Chloropropionyl-L-tyrosine	155[e]	....	+42.1	5.2	13.1	5.2	13.3
DL-Chloropropionyl-L-alanine[f]	157	−51.1	−28.7	7.8	19.8	7.8	19.6

[a] Degrees corrected. [b] In degrees, concentration 2.0%, 2-dcm. polarimeter tube employed. [c] Mixed m.p. 153–176°. [d] Mixed m.p. 66°. [e] Mixed m.p. 134–136°. [f] DL-Chloropropionyl-D-alanine is antipodal, and possesses an $[\alpha]^{24}D$ +51.1° (*c* 2, H_2O) = +28.7° (*c* 2, methanol) and m.p. 157°. The antipodal DL-bromopropionyl-L-alanine and DL-bromopropionyl-D-alanine possessed a m.p. of 173°; $[\alpha]^{24}D$ for the former −41.2° and −26.6° (*c* 2, H_2O and methanol, respectively), and for the latter = +40.9° and +26.5° under the same conditions.

with careful exclusion of moisture. At the end of this time the evolution of HCl gas through the drying tube had ceased. The liquid was distilled between 30 and 50° at 100 mm. pressure, and then fractionated through a 30-cm. unpacked column. The pure acid chloride distilled at 53° under a pressure of 100 mm.

Anal. Calcd. for $C_3H_4OCl_2$: Cl, 55.8. Found (for L-chloropropionyl chloride): Cl, 56.0; $\alpha^{25}D$ +5.8 (*l* = 10 cm.), $[\alpha]^{25}D$ +4.6°, d^{25} 1.2689, $n^{25}D$ 1.4368. Found (for D-chloropropionyl chloride): Cl, 55.8; $\alpha^{25}D$ −5.8 (*l* = 10 cm.), $[\alpha]^{25}D$ −4.6°, d^{25} 1.2682, $n^{25}D$ 1.4370.

L- and D-Chloropropionyl-L-amino Acids.—The L-butyrine, L-norvaline and L-phenylalanine were obtained by enzymatic resolution.[1] The L- and D-chloropropionyl chloride was combined in alkaline solution with glycine, L-alanine or L-butyrine by the usual Schotten–Baumann procedure, the solution acidified, and the acylated amino acids extracted into ethyl acetate. Condensation of the dried extract, followed by careful addition of petroleum ether, yielded in all cases crystalline compounds. Acidification of the reaction mixtures involving L-norvaline and L-phenylalanine caused precipitation of the acylated amino acids. The acylated phenylalanines were recrystallized first from benzene–petroleum ether and then from either aqueous ethanol or aqueous acetone. D-Chloropropionyl-L-phenylalanine was recrystallized first from dilute ethanol and subsequently from ethyl acetate. The L-chloropropionyl-L-norvaline, however, appeared as an oil. It was dissolved in ethyl acetate, the solution treated with Norit and the filtrate condensed to a residual sirup which slowly was transformed during standing for 6 months into a semi-solid.

The acylated tyrosines were prepared by coupling the acid chloride with the free methyl ester of L-tyrosine [$[\alpha]^{25}D$ +25.5° (*c* 2, methanol)] in chloroform solution plus sodium carbonate.[21] On brief standing at −10°, crystallization of the products was completed. They were recrystallized from 4:1 water–ethanol mixtures.

Anal. Calcd. for $C_{13}H_{16}O_4NCl$: C, 54.6; H, 5.6; N, 4.9; Cl, 12.4. Found (for L-chloropropionyl-L-tyrosine methyl ester): C, 54.5; H, 5.6; N, 5.0; Cl, 12.5; m.p. 113°. Found (for D-chloropropionyl-L-tyrosine methyl ester): C, 54.6; H, 5.7; N, 5.0; Cl, 12.5; m.p. 113°. A mixed m.p. of both forms was 83°. The $[\alpha]^{25}D$ for D-chloropropionyl-L-tyrosine methyl ester +21.4° (*c* 2, methanol); the L-chloropropionyl-L-tyrosine methyl ester was too insoluble in this solvent for its rotation to be accurately measured.

The two compounds were saponified in the presence of 1 *N* NaOH at 25° for 1 hour, and the solutions treated with the equivalent amount of 5 *N* HCl. The D-chloropropionyl-L-tyrosine readily crystallized. Under the same conditions, no crystallization of the L-chloropropionyl-L-tyrosine was evident, and consequently its solution was evaporated at low temperature *in vacuo* to dryness, the dried residue extracted with hot acetone, and the filtered acetone extract condensed to a low bulk when crystallization began. Both compounds were purified by crystallization from 4:1 ether–ethanol, and then from 10:1 water–ethanol.

The physical constants of the L- and D-chloropropionyl-L-amino acids are collected in Table IV. Other compounds prepared were acetyl-L-phenylalanine [m.p. 171°, $[\alpha]^{25}D$ +52.5° (*c* 2, ethanol), calcd. N, 6.7, found N, 6.7], and propionyl-L-phenylalanine [m.p. 132°, $[\alpha]^{25}D$ +50.0° (*c* 2, ethanol), calcd. N, 6.4, found N, 6.4]. Chloroacetyl-L-phenylalanine has been described.[3] Comparison of the optical rotation values for DL-chloropropionyl-L-alanine on the one hand with those of L-chloropropionyl-L-alanine and D-chloropropionyl-L-alanine on the other, reveals the essential optical purity of the latter two compounds, for the values for the former are close to the exact mean of the sum of the latter (Table IV).

L- and D-Alanyl-L-amino Acids.—The L- and D-alanyl derivatives of glycine, L-phenylalanine and L-tyrosine, as well as L-alanyl-L-alanine, were part of the peptide collection of the late Dr. Erwin Brand.

D-Alanyl-L-alanine was prepared by the interaction of carbobenzoxy-D-alanine [m.p. 84°, $[\alpha]^{25}D$ +14.1° (*c* 2, glacial acetic acid)] with L-alanine ethyl ester [$[\alpha]^{25}D$ of the HCl salt = −11.4° (*c* 2, 5 *N* HCl)] in a toluene–chloroform mixture, and in the presence of isovaleryl chloride and triethylamine.[22] Using the theoretical quantity of L-alanine ester, the yield of carbobenzoxy-D-alanyl-L-alanine ethyl ester was 87% of the theoretical. When isobutyl chlorocarbonate was used in place of isovaleryl chloride in the presence of excess ester the yield of coupling product was very low; m.p. 89.5°.

Anal. Calcd. for $C_{16}H_{22}O_6N_2$: C, 59.6; H, 6.9; N, 8.7. Found: C, 59.7; H, 7.1; N, 8.7.

Saponification of the ester in NaOH followed by addition of HCl produced a thick yellow oil which ultimately crystallized after rubbing with petroleum ether. The carbobenzoxy-D-alanyl-L-alanine was purified by two crystallizations from ethyl acetate; m.p. 116°.

Anal. Calcd. for $C_{14}H_{18}O_5N_2$: C, 57.1; H, 6.2; N, 9.5. Found: C, 57.1; H, 6.2; N, 9.5.

Hydrogenolysis of the carbobenzoxy group was effected in 1:1 methanol–water, and the free peptide was isolated and crystallized from water and hot ethanol. Its $[\alpha]^{25}D$ of −71.1° (*c* 2, H_2O) is practically identical with that of +71.2° described by Erlanger and Brand for the antipodal L-alanyl-D-alanine.[23]

L- and D-alanyl-L-butyrine and L- and D-alanyl-L-norvaline were prepared by substantially the same procedure, using

(21) E. Fischer and W. Schrauth, *Ann.*, **354**, 21 (1907).

(22) J. R. Vaughan and R. L. Osato, THIS JOURNAL, **73**, 5553 (1951); **74**, 676 (1952).

(23) B. F. Erlanger and E. Brand, THIS JOURNAL, **73**, 3508 (1951).

isovaleryl chloride as the condensation reagent. L-Butyrine ethyl ester and L-norvaline ethyl ester hydrochlorides [$[\alpha]^{25}D$ +6.1° and +9.5° (*c* 2, 5 *N* HCl), respectively] were used in the synthesis of the four dipeptides. The over-all yields varied from 65 to 80% of the theoretical. Data on the intermediates are as follows.

Ethyl carbobenzoxy-L-alanyl-L-butyrinate: m.p. 77°; calcd. N, 8.3; found N, 8.3. Ethyl carbobenzoxy-D-alanyl-L-butyrinate: m.p. 93°, calcd. N, 8.3; found N, 8.8. Carbobenzoxy-L-alanyl-L-butyrine: m.p. 154°; calcd. N, 9.1; found N, 9.3. Carbobenzoxy-D-alanyl-L-butyrine: m.p. 141°; calcd. N, 9.1; found N, 9.2.

Ethyl carbobenzoxy-L-alanyl-L-norvalinate: m.p. 108°; calcd. N, 8.0; found N, 8.0. Ethyl carbobenzoxy-D-alanyl-L-norvalinate: m.p. 90°; calcd. N, 8.0; found N, 8.1. Carbobenzoxy-L-alanyl-L-norvaline: m.p. 145°; calcd. N, 8.7; found N, 8.7. Carbobenzoxy-D-alanyl-L-norvaline: m.p. 151°; calcd. N, 8.7; found N, 8.7.

The carbobenzoxy peptides were hydrogenated as usual in the presence of palladium black, and the free peptides recrystallized from ethanol–H_2O. The analytical data are collected in Table V. Carbobenzoxy-L-alanyl-L-phenylalanine and carbobenzoxy-D-alanyl-L-phenylalanine were prepared by the interaction of the respective carbobenzoxyalanine with L-phenylalanine ethyl ester in the presence of isovaleryl chloride and triethylamine, and the resulting coupling product subsequently saponified and acidified. The compounds were recrystallized first from ethyl acetate and petroleum ether and then from acetone and petroleum ether; m.p. for the former compound was 122°, for the latter compound 74°. Bergmann and Fruton reported a m.p. of 56–58° for the former compound.[24]

TABLE V

PHYSICAL CONSTANTS OF L- AND D-ALANYL-L-AMINO ACIDS

Compound	$[\alpha]^{25}D$[a]	Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
D-Alanyl-L-alanine	−71.1	45.0	7.6	17.5	44.7	7.4	17.3
L-Alanyl-L-butyrine	− 8.0	48.3	8.1	16.1	48.4	8.4	16.2
D-Alanyl-L-butyrine	−52.8	48.3	8.1	16.1	48.2	8.2	16.2
L-Alanyl-L-norvaline	− 5.0	51.0	8.6	14.9	51.1	8.4	14.8
D-Alanyl-L-norvaline	−47.4	51.0	8.6	14.9	50.9	8.4	14.8

[a] In degrees, *c* 2, H_2O.

Anal. Calcd. for $C_{20}H_{22}O_5N_2$: C, 64.9; H, 6.0; N, 7.6. Found (for carbobenzoxy-L-alanyl-L-phenylalanine): C, 64.8; H, 5.8; N, 7.6. Found (for carbobenzoxy-D-alanyl-L-phenylalanine): C, 64.6; H, 6.1; N, 7.5.

Enzymatic Studies.—The hydrolysis of all substrates studied was followed by the manometric ninhydrin–CO_2 procedure. A single lyophilized preparation of renal acylase I was employed.[1] The enzyme solution in 0.01 *M* phosphate buffer at *p*H 7.0 was prepared fresh daily. Digests consisted of 1 ml. of 0.1 *M* phosphate buffer at *p*H 7.0, 1 ml. of 0.025 *M* neutralized substrate, and 1 ml. of appropriately diluted enzyme solution. The period of incubation, which was at 37°, varied up to 120 minutes. As with all substrates studied with acylase I under these conditions, zero-order kinetics were obtained. Rates were calculated from the initial linear portion of the time curves, and were expressed as micromoles of substrate hydrolyzed per hour per mg. of protein N. All rate values given, both with acylase I and with pancreatic carboxypeptidase, were the average of from 4 to 8 separate determinations with an average variation of less than 10%.

Studies with pancreatic carboxypeptidase were conducted with a crystalline Armour preparation. The enzyme was suspended in chilled 5% LiCl solution and filtered clear after 18 hours of standing at 5°. Digests consisted of 1 ml. of veronal buffer at *p*H 7.45, 1 ml. of 0.025 *M* neutralized substrate and 1 ml. of appropriately diluted enzyme solution. Rates were expressed in terms of the first-order velocity constant per mg. of protein N per ml. of digest. The experimental conditions were different from those frequently employed which include an initial substrate concentration of 0.05 *M* and an incubation temperature of 25°.[14,24] The present results are therefore difficult to compare with those in the literature. Thus, Bergmann and Fruton report a rate value of 7.3 for the action of carboxypeptidase on carbobenzoxy-L-alanyl-L-phenylalanine,[24] a value subsequently altered to 11.3 by Neurath and Schwert.[14] The present value of 71.0 (Table III) obtained for this compound with an initial substrate concentration of 0.008 *M*, divided by the ratio of 0.05 to 0.008 or close to 6, would yield a value of 11.8. Inasmuch as the temperature at which the present and the cited experiments were performed was quite different (by 12°), and because there is no reason to expect that the proteolytic coefficients would be linear in the substrate concentration over so wide a range of concentration, the comparison made is quite approximate and serves only to reveal a similar order of magnitude.

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(24) M. Bergmann and J. S. Fruton, *J. Biol. Chem.*, **145**, 247 (1942).

[CONTRIBUTION FROM THE VIRUS LABORATORY AND THE HORMONE RESEARCH LABORATORY, DEPARTMENT OF BIOCHEMISTRY UNIVERSITY OF CALIFORNIA, BERKELEY]

A General Micromethod for the Stepwise Degradation of Peptides

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The phenyl isothiocyanate method for the stepwise degradation of peptides from the amino end has been adapted to a microscale and modified in such a manner as to be applicable to peptides containing all natural amino acids, with the possible exception of cystine. This is achieved by following with the spectrophotometer the formation of the phenylthiohydantoins in strong aqueous acid and thus avoiding unnecessary exposure to acid.

The application of the phenyl isothiocyanate (PTC) method[2] to the stepwise degradation of peptides and proteins was greatly facilitated when it was shown that the phenylthiohydantoin (PTH) derivative of the N-terminal amino acid could be released in aqueous acid solution.[3] Several different conditions have been used for the aqueous acid cleavage of phenylthiohydantoins from the corresponding phenylthiocarbamyl (PTC-) peptides. Thus Ottesen and Wollenberger[4] employed 0.1 *N* HCl at 75° for the successive removal of N-terminal amino acids from the hexapeptide (ala·gly·val·asp·ala·ala) released during the transformation of ovalbumin to plakalbumin. Under these conditions, however, the very labile bond between aspartic acid and alanine was also hydrolyzed during the course of the second step in the degradation. Christensen[5] found extensive non-specific splitting

(1) Aided by a grant from the National Foundation for Infantile Paralysis.

(2) P. Edman, *Acta Chem. Scand.*, **4**, 283 (1950).

(3) H. Fraenkel-Conrat and J. Fraenkel-Conrat, *ibid.*, **5**, 1409 (1951).

(4) M. Ottesen and A. Wollenberger, *Compt. rend. trav. lab. Carlsberg, ser. chim.*, **28**, 463 (1953).

(5) H. N. Christensen, *ibid.*, **28**, 265 (1953).