[CONTRIBUTION FROM THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Studies on Diastereoisomeric α-Amino Acids and Corresponding α-Hydroxy Acids. VII. Influence of β-Configuration on Enzymic Susceptibility

BY MILTON WINITZ, LEAH BLOCH-FRANKENTHAL,[1] NOBUO IZUMIYA, SANFORD M. BIRNBAUM, CARL G. BAKER AND JESSE P. GREENSTEIN

RECEIVED DECEMBER 8, 1955

A correlation between the β-configuration of α,β-diasymmetric amino acids and the susceptibility exhibited by these compounds to ten different *ophio*-L-amino acid oxidases and two different D-amino acid oxidases has been noted. An α-D-β-D-configuration (for D-amino acid oxidases) and an α-L-β-L-configuration (for L-amino acid oxidases) led to a more marked enzymic susceptibility than their corresponding diastereomers. The four optical antipodes of threonine and isoleucine served as substrates in the aforementioned. Extension of such relationship to the four stereoisomers of O-methylthreonine, as well as those of α-hydroxy-β-methylvaleric acid, has been effected. The racemic O-methylthreonine, as its acetyl and chloroacetyl derivatives, and O-methylallothreonine, as its formyl derivative, were resolved by a general enzymic resolution procedure developed in this Laboratory whereas nitrous acid deamination of the four optically pure isomeric isoleucines provided the corresponding α-hydroxy-β-methylvaleric acids. Oxidation rates of the former four enantiomorphs were determined with D- and L-amino acid oxidases, as were the hydrolytic rates of the formyl, acetyl and chloroacetyl derivatives with renal acylase I. Correspondingly, L-hydroxy acid oxidase (rat kidney) gave oxidation rates for the stereoisomeric L-α-hydroxy-β-methylvaleric acids, as well as eleven other L-α-hydroxy acids. The corresponding D-α-hydroxy acids were prepared for comparison purposes. In addition, the susceptibility to hog kidney aminopeptidase of several dipeptides with diasymmetric amino acid residues on the terminal amino end has been determined. The correlation of β-configuration with enzymic susceptibility is discussed.

Past studies on the optical specificity exhibited by such enzymes as the amino acid oxidases[2a] and decarboxylases,[2b] the transaminases[3] and the peptidases,[4] have been confined almost exclusively to the α-asymmetric center of the substrate amino acid, or pertinent derivatives thereof. Since some 20% of the various amino acids which compose proteins contain two centers of optical asymmetry[5] and since the ω-center of asymmetry of these amino acids appears, in all instances, to be restricted to a definite configuration, then the influence of such configuration on the enzymic susceptibility exhibited by the amino acid becomes of primary consideration. A previous communication[6] from this Laboratory noted a correlation between the β-configuration of α,β-diasymmetric amino acids and the susceptibility of these compounds (or their acyl derivatives) to the action of L- and D-amino acid oxidase or of the α-L-directed renal acylase I. Substrates with an α-D-β-D-configuration and an α-L-β-L-configuration showed a markedly greater susceptibility to the enzymic action of D-amino acid oxidase and of L-amino acid oxidase or renal acylase I, respectively, than the corresponding diastereomer wherein the β-carbon atom was inverted. Extension of this relationship to compounds derived from the four stereoisomeric forms of threonine and isoleucine, as well as to several other enzyme systems, forms the basis of the current report.

Hydrolytic Susceptibility of O-Methylthreonine Derivatives to Renal Acylase I.—In the attempt to ascertain the general applicability of the phenomenon that an L-configuration at the β-asymmetric center of primarily α-L-directed enzymes is more conducive to enzymic action than a β-D-configuration, whereas α-D-directed enzymes prefer a β-D-configuration,[6] several derivatives of the stereoisomers of threonine and isoleucine were prepared and their enzymic susceptibilities tested. Because of their close structural relationship to the isomeric isoleucines, as well as to the isomeric threonines, the optical antipodes of O-methylthreonine and O-methylallothreonine were among the first to be synthesized.

In Fig. 1, the L-antipodes of O-methylthreonine, threonine and isoleucine are presented by their conventional Fischer formulas. Examination reveals that O-methyl-L-threonine may be considered as closely related to L-isoleucine, in addition to L-threonine from which it may be derived. Thus, either a substitution of the hydroxyl-hydrogen atom of L-threonine with a methyl group or a replacement of the C_4-methylene group of L-isoleucine with an oxygen atom both yield this compound. However, the β-asymmetric center of L-threonine and of L-isoleucine have been shown to be configurationally related to D-[7] and to L-glyceraldehyde,[6,8,9] respectively. The configurational relationship of the β-asymmetric center of the O-methylthreonines to compounds of established configuration therefore is somewhat ambiguous when based solely on structural considerations. Thus the stereochemical discrimination of these compounds or suitable derivatives thereof, shown by the susceptibility to the action of various enzyme systems of established optical specificity, were studied.

(1) Fellow of the American Association of University Women, on leave from the Hebrew University, Jerusalem. Aid from the National Science Foundation is also gratefully acknowledged.

(2a) H. A. Krebs, "The Enzymes," Vol. II, Academic Press, Inc., New York, N. Y., 1951, p. 499.

(2b) H. Blaschko, *Advances in Enzymol.*, **5**, 68 (1945); E. F. Gale, *ibid.*, **6**, 1 (1946).

(3) H. A. Krebs, *Symposia of the Biochemical Society*, No. 1 (1948).

(4) M. Bergmann and J. S. Fruton, *Advances in Enzymol.*, **1**, 63 (1941).

(5) The L-amino acids thus far demonstrated in proteins which contain two centers of optical asymmetry include threonine, hydroxyproline, isoleucine, cystine and hydroxylysine. Demonstration of an L-configuration for the β-asymmetric center of L-isoleucine[6] and a D-configuration for the ω-center of asymmetry of L-threonine[7] and L-hydroxyproline (A. Neuberger, *J. Chem. Soc.*, 429 (1945)) has been made, whereas the configuration of the δ-asymmetric center of L-hydroxylysine remains uncertain at the present time.

(6) M. Winitz, S. M. Birnbaum and J. P. Greenstein, THIS JOURNAL, **77**, 3106 (1955).

(7) C. E. Meyer and W. C. Rose, *J. Biol. Chem.*, **115**, 721 (1936).

(8) S. Stälberg-Stenhagen and E. Stenhagen, *Arkiv. Kemi Mineral. Geol.*, **24B**, 1 (1947).

(9) J. Trommel, *Proc. Koninkl. Ned. Akad. Wetenschap.*, Series B, **56**, 272 (1953); *ibid.*, **57**, 364 (1954).

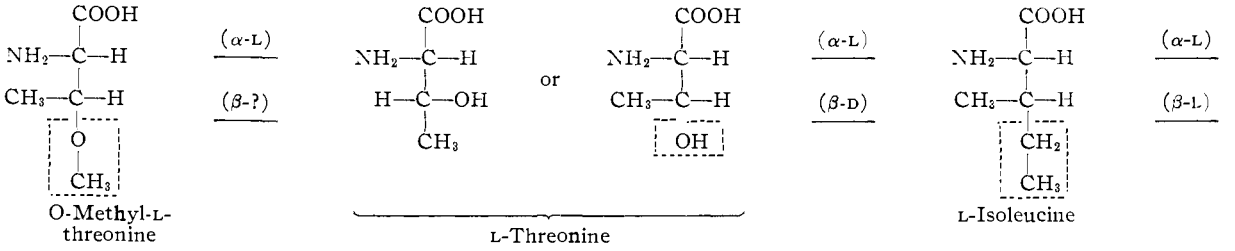

Fig. 1.—Fischer configurations of diasymmetric amino acids.

The O-methyl-DL-threonine[10] and O-methyl-DL-allothreonine[11] used in this investigation were prepared from crotonic acid according to the procedure of Carter and co-workers. The former compound, as its acetyl and chloroacetyl derivatives, and the latter, as its formyl derivative, were resolved according to the general enzymic resolution procedure developed in this Laboratory.[12] Optical rotation values for the enantiomorphs were equal and opposite within the limits of experimental error. These values were in complete agreement with those reported by West and Carter[13] for the optical antipodes of O-methylthreonine, which were obtained *via* a brucine resolution of the formyl derivative. The previously unresolved O-methylallothreonine enantiomorphs showed specific rotation values of +30.5° and −29.8° (*c* 4% in 5 *N* HCl) for the L- and D-forms, respectively. These values are in accord with the +30.5° value obtained for the specific rotation of a preparation of O-methyl-L-allothreonine secured *via* methoxylation of acetyl-L-allothreonine with methyl iodide and silver oxide followed by acid hydrolysis. For each of the four stereoisomeric forms here resolved, routine purity determinations with L- and D-amino acid oxidase[14] indicated a level of possible contamination of one isomer by the other which was less than 1 part in 1,000.

The formyl, acetyl and chloroacetyl derivatives of the O-methyl-DL- and O-methyl-DL-allo-forms of threonine were subjected to the hydrolytic action of the α-L-directed renal acylase I.[15] Preparation of this enzyme from homogenates of hog kidney has been described previously.[15] The rates, as revealed in Table I, are described in terms of micromoles of substrate hydrolyzed per hour per mg. of protein nitrogen.

A comparison of the values given in Table I indicates that the expected order of rates, chloroacetyl > acetyl > formyl, was found for both diastereomeric forms of the amino acid.[16] The hydrolytic rates exhibited by the acyl derivatives of O-methylallothreonine are approximately 100-fold faster than those of the corresponding O-methylthreonine derivatives. A β-L-configuration was suggested for L-O-methylallothreonine and, conversely, a β-D-configuration indicated for its α-L-diastereomer. This suggestion was based on the previously observed preference of renal acylase I for the acyl derivatives of L-isoleucine, L-allothreonine and L-allophenylserine, all of which have a β-L-configuration, rather than for their corresponding α-L-diastereomeric forms.[6] A comparable preference has been indicated by Smith and Spackman[17]; the leucine aminopeptidase-catalyzed hydrolysis of L-isoleucinamide proceeded at a faster rate than that of L-alloisoleucinamide. The following work was undertaken to determine whether or not these same preferences held for the amino acid oxidases.

TABLE I

SUSCEPTIBILITY OF VARIOUS DERIVATIVES OF THE DIASTEREOMERIC L-O-METHYLTHREONINES TO HOG KIDNEY ACYLASE I

Amino acid[a]	Rate of hydrolysis[b] Formyl	Acetyl	Chloroacetyl
O-Me-threonine	<0.5	8.3	25
O-Me-allothreonine	55	730	3320

[a] The acyl-DL-amino acid was here used. Since only the acyl-L-amino acid is hydrolyzed, the substrate concentration was considered on the basis of the hydrolyzable L-form only. [b] In terms of micromoles of substrate hydrolyzed at 38° per hour per mg. of protein N; all digestions conducted in phosphate buffer at *p*H 7.0.

Susceptibility of Diastereomers to Amino Acid Oxidase Action.—As is revealed in Tables II and III, the oxidation rates of the L- and L-allo-antipodes of isoleucine, threonine and O-methylthreonine were determined with ten different *ophio*-L-amino acid oxidases, whereas for the corresponding D-stereoisomers, preparations of hog and sheep kidney D-amino acid oxidase were employed. The enzymic determination with an L-amino acid oxidase was conducted as before.[12]

Examination of Table II indicates that for each of the L-amino acid oxidases tested, the substrates L-isoleucine and L-allothreonine, each of which has a β-L-configuration, were oxidized at a significantly more rapid rate than their corresponding β-D-stereoisomers. Such results are in agreement with previously observed preference of α-L-directed oxidases for substrates with a β-L-configuration.[6] In the case of the steroisomeric O-methylthreonines, however, the results were entirely unexpected when interpreted in the light of the hydrolytic data obtained by the action of the α-L-directed renal acylase I on the acyl derivatives of these same amino acids. Thus, unlike acylase I, where a marked preference was revealed for the β-asymmetric center of

(10) H. E. Carter and H. D. West, "Organic Syntheses," Coll. Vol. III, John Wiley and Sons, Inc., New York, N. Y., 1955, p. 813.

(11) H. D. West, G. S. Krummel and H. E. Carter, *J. Biol. Chem.*, **122**, 605 (1937–1938).

(12) J. P. Greenstein, S. M. Birnbaum and M. C. Otey, *J. Biol. Chem.*, **204**, 308 (1953); J. P. Greenstein, *Advances in Protein Chemistry*, **9**, 122 (1954).

(13) H. D. West and H. E. Carter, *J. Biol. Chem.*, **119**, 109 (1937); **122**, 611 (1938).

(14) A. Meister, L. Levintow, R. Kingsley and J. P. Greenstein, *ibid.*, **192**, 535 (1951).

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

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

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

TABLE II

RATES OF OXIDATION[a] OF DIASTEREOMERIC L-AMINO ACIDS WITH VARIOUS *ophio*-OXIDASES

L-Amino acid oxidase	L-Threonine, μmole	L-Allo-threonine, μmole	L-Isoleucine, μmole	L-Alloiso-leucine, μmole	O-Me-L-Threonine, μmole	O-Me-L-Allothreonine, μmole
Crotalus adamanteus	0	0.90	71.00	0.80	1.12	0.39
Crotalus basiliscus	0.45	0.83	8.71	0.78	0.14[b]	0
Bothrops jararaca	0	1.17	19.88	1.03	0.26	0.10
Naja hanna	0	4.40	38.90	3.79	0.59	0.25
Agkistrodon contortrix	0	1.42	8.41	1.31	0.52	0
Naja flava	0.23	0.38	3.20	0.11	0.08[b]	0.06
Crotalus terrificus	0.63	4.04	41.36	3.09[b]	1.42	0.65
Crotalus h. atricandatus	0.55	2.25	16.35	1.20	0.37	0
Crotalus h. horridus	0	0.91	5.59	0.66	0.20[b]	0
Bungarus yasciatus	0	0.36	0.81	0.08	0	0

[a] In terms of micromoles of oxygen consumed per hr. per mg. of N at 38°. [b] Rate determined after an initial lag period of 20 min.

TABLE III

INFLUENCE OF THE β-ASYMMETRIC CENTER ON THE SUSCEPTIBILITY OF THE α-ASYMMETRIC CENTER OF DIASTEREOMERIC AMINO ACIDS TO D-AMINO ACID OXIDASES

D-Amino acid	β-Carbon	Rate of oxidation[a] Sheep kidney	Hog kidney
Isoleucine	D	9.3	14.8
Alloisoleucine	L	5.4	5.9
Allothreonine	D	9.7	2.6
Threonine	L	1.4	0.3
O-Me-threonine	?	5.0	5.0
O-Me-allothreonine	?	2.3	4.5

[a] In terms of micromoles of oxygen consumed per hr. per mg. N at 38°.

O-methyl-L-allothreonine, the *ophio*-oxidases exerted a more pronounced action on O-methyl-L-threonine. With enzyme systems of this latter type, such preference was consistent not only for all the ten L-amino acid oxidases tested, but also for the D-amino acid oxidases, the data for which are depicted in Table III.

Table III indicates that the oxidation, with sheep and hog kidney oxidase, of D-isoleucine and D-allothreonine, wherein the β-configuration is D, proceeds at a faster rate than the corresponding α-D-β-L-diastereomers. These results are completely compatible with the concept that for α-D-directed enzymes, a β-D-configuration is more conducive to enzymic action than a β-L-configuration.[6] Thus, the more appreciable oxidation rate exhibited by O-methyl-D-threonine, than by the D-allo-form, in a D-amino acid oxidase catalyzed system, is entirely consistent with and predictable from the L-amino acid oxidase data.

Because of the discrepancy between the results with the renal acylase I system and the oxidase system when the isomeric O-methylthreonines were the substrates, it was of interest to determine whether the above concept applied to other peptidase systems. Thus, the following work with a highly purified hog kidney aminopeptidase[18] was carried out.

Effect of β-Configuration on Susceptibility to Aminopeptidase.—Prior knowledge concerning the specificity of aminopeptidase[18] dictated that investigation of the influence of β-configuration on the susceptibility of the substrate to this enzyme required synthetic dipeptides wherein the L- and L-allo-forms of threonine and isoleucine were the residues on the terminal amino end of the peptide. It was also deemed advisable that the peptides contain a D-amino acid residue on the terminal carboxyl end of the molecule, in order to limit the potential action of other peptidases which might contaminate the aminopeptidase preparation and thus confound the data. For this purpose, D-alanine was the residue of choice. The peptides were prepared *via* a coupling of the carbobenzoxyl-ated diasymmetric amino acid with D-alanine benzyl ester according to either the mixed anhydride procedure of Vaughan and Osato[19] or the "carbodiimide" method of Sheehan and Hess.[20] Subsequent hydrogenolysis of the carbobenzoxy dipeptide benzyl ester, in a palladium black catalyzed system, yielded the corresponding free dipeptide.

The previously reported solubilized hog kidney amino acid peptidase, available in a high degree of purity, was used. It is equally active toward substrates with a D- or an L-amino acid residue on the terminal carboxyl end of a dipeptide. The rates are given in Table IV in terms of micromoles of substrate hydrolyzed per hr. per mg. of protein nitrogen.

TABLE IV

INITIAL RATES OF HYDROLYSIS OF SEVERAL DIPEPTIDES WITH HOG KIDNEY AMINOPEPTIDASE

Dipeptide	β-Configuration	Rate in μmoles[a]
L-Allothreonyl-D-alanine	L	1,900
L-Threonyl-D-alanine	D	630
L-Isoleucyl-D-alanine	L	198
L-Alloisoleucyl-D-alanine	D	530
D-Alloisoleucyl-D-alanine	L	0
Glycyl-D-alanine	...	19,200

[a] Rate in terms of micromoles of substrate hydrolyzed per hr. per mg. of protein nitrogen.

Examination of Table IV reveals that the presence of a D-amino acid residue on the amino end of a peptide is characterized by a markedly decreased susceptibility to enzymic action. Therefore, the lack of measurable activity observed with D-alloisoleucyl-D-alanine is compatible with the data previously obtained[18] for the reduced hydrolytic activity

(18) D. S. Robinson, S. M. Birnbaum and J. P. Greenstein, *J. Biol. Chem.*, **202**, 1 (1953).

(19) J. R. Vaughan, Jr., and R. Osato, THIS JOURNAL, **74**, 676 (1952).

(20) J. C. Sheehan and G. P. Hess, *ibid.*, **77**, 1067 (1955).

toward peptides wherein an L-alanine residue is replaced by a D-alanine residue at the terminal amino position. All peptides with an L-amino acid residue at this position were susceptible to the hydrolytic action of the peptidase but to a markedly lesser extent than glycyl-D-alanine, thereby invoking the possibility of steric effects. Thus, L-allothreonyl-D-alanine, wherein the diasymmetric residue contains a β-L-asymmetric carbon atom, exhibited a threefold greater susceptibility to hydrolysis than the diastereomeric L-threonyl-D-alanine, in which the β-configuration is D. However, the data secured with the "isoleucine-containing" peptides indicated that L-alloisoleucyl-D-alanine, with a β-D-configuration, was more reactive than its β-L-diastereomer. These results, reinforced by the previously discussed inconsistencies observed between acylase I and the amino acid oxidases, strongly suggest that the effect of a β-configuration on action by hydrolytic enzymes is not open to any simplified explanation. In the absence of further data, therefore, interpretations of the effect of secondary asymmetric centers on peptidase action must, of necessity, be deferred.

Specificity of a Hog Kidney α-Hydroxy Acid Oxidase.—In view of the preferential action of amino acid oxidases based on the configuration of the secondary asymmetric center of α,β-diasymmetric amino acids, it became of interest to examine substrates other than amino acids. A rat kidney α-hydroxy acid oxidase, previously investigated in this Laboratory,[21] was deemed as an appropriate enzyme system of study. Earlier reports by Blanchard, Green, Nocito and Ratner[22] indicated that the L-α-hydroxy acid oxidase and L-amino acid oxidase activities of rat kidney resided in the same enzyme. That lactic acid dehydrogenase activity was not identical with this enzyme system was later shown by Iselin and Zeller.[23] An earlier finding by Meyerhof and Lohmann[24] that rat kidney slices exhibited activity toward both L- and D-lactic acid was more recently extended[21] to include a large number of optically active D- and L-α-hydroxy acids with both the supernatant and particulate fractions of rat kidney homogenates. Identification of the D-activity with the particulate fraction and the L-activity with both the particulate and the supernatant fractions was made.

Synthesis of the optical isomers of α-hydroxy-β-methylvaleric acid was effected by the nitrous acid deamination of the corresponding optical antipode of isoleucine.[25] That such conversion of an α-amino acid to the corresponding α-hydroxy acid proceeds without inversion of configuration or appreciable racemization has been amply demonstrated.[26-28] Thus, the configuration of each of the α-hydroxy-β-methylvaleric acid isomers can be considered identical to that of the parent amino acid from which it was derived.

In the initial studies, the above α-hydroxy acids were first tested for their susceptibility to oxidation by rat kidney homogenates. Subsequent separation of the supernatant from particulates gave results in agreement with the prior report[21] of D-hydroxy acid oxidase activity in the latter fraction and L-activity in both fractions. However, heat treatment of the particulate fraction resulted in a several-fold increase in its L-activity and a diminution or virtual destruction of the D-activity on prolonged heating. Thus, heat treatment at 60° for 15 and 30 minutes gave a four- and fivefold increase, respectively, in L-oxidase activity and an eightfold decrease in D-activity for the longer period of heating. Similar treatment resulted in no change in the L-activity of the supernatant. This implies the association with the particulate fraction of a heat-labile L-inhibitor. Aside from these initial observations, studies on neither the D-oxidase nor on the cause of inhibition were pursued any further.

The ratios of the oxidation rates observed with various L-α-hydroxy acids suggested that the L-activity of the supernatant fraction represented a soluble form of the same enzyme found in the particulates. Because of its somewhat greater, as well as less variable activity, studies on the effect of β-configuration on enzymic action were confined to the oxidase which resided in the supernatant fraction. Rates of oxidation for the L- and L-allo-forms of α-hydroxy-β-methylvaleric acid were found, however, to be of like magnitude, and therefore apparently independent of the optical configuration of the β-asymmetric center. Further investigation with other α-hydroxy acids revealed that with the exception of L-lactic acid, which showed a somewhat lower activity, the rates of oxidation of all the L-α-hydroxy acids investigated which contained aliphatic side chains were of comparable magnitude (Table V). Since the L-oxidase demonstrated little aliphatic side-chain specificity,

TABLE V

ENZYMIC SUSCEPTIBILITY OF VARIOUS L-α-HYDROXY ACIDS TO THE SUPERNATANT FRACTION OF RAT KIDNEY

L-α-Hydroxy acid	Rate of oxidation[a]
α-Hydroxy-β-methylvaleric	3.6
Allo-α-hydroxy-β-methylvaleric	3.8
Lactic	1.1
α-Hydroxybutyric	2.8
α-Hydroxyvaleric	4.8
α-Hydroxycaproic	4.6
α-Hydroxyisovaleric	4.8
α-Hydroxyisocaproic	3.2
α-Hydroxy-β-phenylpropionic	2.4
Malic	0
α-Hydroxyglutaric	0
Isocitric	0
Alloisocitric	0

[a] In a typical enzymic run, each vessel contained 1.50 ml. of enzyme (20 mg. of lyophilized powder per ml.), 0.10 ml. of 2-octanol, 0.90 ml. of 0.067 *M* sodium phosphate (*p*H 7.2) and 0.50 ml. of 0.03 *M* substrate. Incubation was at 38°. Rates were expressed in terms of micromoles of oxygen consumed per hr. per mg. of protein nitrogen.

(21) C. G. Baker, *Arch. Biochem. Biophys.*, **41**, 325 (1952).

(22) M. Blanchard, D. Green, V. Nocito and S. Ratner, *J. Biol. Chem.*, **155**, 421 (1944); **161**, 583 (1945).

(23) B. Iselin and E. A. Zeller, *Helv. Chim. Acta*, **29**, 1508 (1946).

(24) O. Meyerhof and K. Lohmann, *Biochem. Z.*, **171**, 421 (1926).

(25) J. P. Greenstein, L. Levintow, C. G. Baker and J. White, *J. Biol. Chem.*, **188**, 647 (1951).

(26) P. Brewster, F. Hiron, E. D. Hughes, C. K. Ingold and P. A. D. S. Rao, *Nature*, **166**, 178 (1950).

(27) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1953.

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

it could therefore hardly be expected to discriminate between the more subtle structural differences imposed by optical considerations. That the enzyme does incorporate some side-chain specificity, however, is evidenced by the complete resistance of substrates with more than one carboxyl group, *i.e.*, L-malic acid, L-α-hydroxyglutaric acid, L-isocitric acid and L-alloisocitric acid, to oxidation under the experimental conditions employed.

Experimental

I. Resolution and Derivatives of O-Methylthreonine. Acetyl-O-methyl-DL-threonine.—Formyl-O-methyl-DL-threonine was prepared according to the procedure of Carter and West[10] and the free racemic amino acid secured upon subsequent hydrolysis with hydrogen bromide, followed by treatment with ammonia. The acetyl derivative was prepared, in the usual manner, by the alternate and portionwise addition of 50 ml. of 2 *N* LiOH and 6.1 g. of acetic anhydride to a cold solution of the 6.7 g. of O-methyl-DL-threonine (previously neutralized with 2 *N* LiOH). This was followed by acidification to congo red with 5 *N* HCl and subsequent evaporation to dryness at 40° under reduced pressure. The residue was taken up in 10 ml. of water and extracted several times with ethyl acetate (350 to 400 ml. in all was used). After drying with anhydrous sodium sulfate, the ethyl acetate layer was evaporated to dryness in a jet of air and the residue taken up in about 50 ml. of boiling acetone, filtered while hot and set at 4° overnight. The crystals were filtered over suction and dried *in vacuo;* yield 2–3 g., m.p. 152–153°.

Anal. Calcd. for $C_7H_{13}O_4N$: C, 48.0; H, 7.5; N, 8.0. Found: C, 48.1; H, 7.4; N, 8.0.

Chloroacetyl-O-methyl-DL-threonine.—This was prepared in a manner analogous to that employed with the acetyl derivative except that sodium hydroxide was employed as the base and chloroacetyl chloride as the acylating agent. After acidification with HCl to congo red the reaction mixture was extracted several times with ethyl acetate, the ethyl acetate layer dried with anhydrous sodium sulfate and then evaporated to dryness *in vacuo*. The crystalline residue was first recrystallized from acetone by the addition of petroleum ether and then twice from the minimal amount of boiling water; m.p. 113–115°.

Anal. Calcd. for $C_7H_{12}O_4NCl$: C, 40.1; H, 5.8; N, 6.7; Cl, 16.9. Found: C, 40.0; H, 5.6; N, 6.8; Cl, 17.1.

Enzymic Resolution of Acetyl- and Chloroacetyl-O-methyl-DL-threonine.—Resolution of the acetyl and chloroacetyl derivatives of O-methyl-DL-threonine was effected in a manner analogous to that employed previously with isovaline.[29] The optical antipodes obtained showed values of $[\alpha]^{25}D$ −38.0° (*c* 4% in water) and −13.5° (*c* 2% in 5 *N* HCl) for the L-isomer and $[\alpha]^{25}D$ +38.5° (*c* 4% in water) and +14.0° (*c* 2% in 5 *N* HCl) for the D-form. West and Carter[13] have reported values of $[\alpha]D$ −37.8° and +38.2°, in water, for the L- and D-antimers, respectively.

II. Resolution and Derivatives of O-Methylallothreonine. Formyl-O-methyl-DL-allothreonine.—O-Methyl-DL-allothreonine was prepared from crotonic acid according to the procedure of West, Krummel and Carter[11] and subsequently formylated as prescribed by Pfister and co-workers.[30] The m.p. found for this compound was in agreement with the reported value of 153–154°.

Acetyl-L-allothreonine.—Acetylation of 23.8 g. of L-allothreonine with alternating portions of acetic anhydride and sodium hydroxide was effected in a manner analogous to that described for acetyl-O-methyl-DL-threonine. After acidification to congo red, the solution was evaporated to dryness *in vacuo*. The residue was extracted with nine 100-ml. portions of boiling acetone and the pooled acetone fractions blown to dryness in a jet of dry air. The residue was dissolved in 60 ml. of hot absolute alcohol and the solution set at 4° overnight. The crystals (16.5 g.) of melting point 153–154° were filtered. Two further crops of crystals amounting to 5.7 g. were obtained by partial evaporation of the filtrate.

Anal. Calcd. for $C_6H_{11}O_4N$: C, 44.7; H, 6.8; N, 8.7. Found: C, 44.4; H, 7.0; N, 8.7.

Acetyl-O-methyl-L-allothreonine.—This compound was prepared according to the procedure developed by Synge[31] for the O-methylation of hydroxyamino acids. A yield of colorless crystals (m.p. 153–155°) amounting to 1.7 g. was secured.

Anal. Calcd. for $C_7H_{13}O_4N$: C, 48.0; H, 7.5; N, 8.0. Found: C, 48.0; H, 7.4; N, 8.0.

Chloroacetyl-O-methyl-DL-allothreonine.—This compound was prepared in the same manner as the corresponding O-methyl-DL-threonine derivative, m.p. 108–109.5°.

Anal. Calcd. for $C_7H_{12}O_4NCl$: C, 40.1; H, 5.8; N, 6.7; Cl, 16.9. Found: C, 40.1; H, 5.6; N, 6.7; Cl, 17.2.

Enzymic Resolution of Formyl-O-methyl-DL-allothreonine.—Twenty-one and one-half grams (0.133 mole) of formyl-O-methyl-DL-allothreonine was dissolved in 1 liter of water and the solution adjusted to *p*H 7.2 by the addition of 2 *N* LiOH. One gram of acylase I powder[15] was dissolved in the solution and water added to bring the volume to 1330 ml. (0.1 *M* concentration of the racemic compound). After the addition of a few drops of toluene, the reaction mixture was incubated at 38° and the enzymic hydrolysis of the substrate followed by the usual manometric ninhydrin-CO_2 procedure. After a digestion period of about 10 hr., analyses on an aliquot of the digest revealed that hydrolysis of the compound had proceeded to 50%. The digest was incubated 12 hr. longer when analysis again revealed a hydrolysis of 50%. After the addition of acetic acid to *p*H 6, the protein was removed by filtration with the aid of Norit. The filtrate was concentrated to a sirup at 40° *in vacuo* and approximately 20 volumes of acetone added thereto. Upon cooling at −15°, the oil which separated underwent partial hardening. Separation of the solvent by decantation, succeeded by replacement with fresh acetone and standing at −15° overnight, resulted in the formation of crystals, 21 g. of which was secured by filtration. The crystals were taken up in hot absolute ethanol and the L-form of the amino acid obtained after partial concentration of this solution *in vacuo*. L-O-Methyl-L-allothreonine, secured by filtration and recrystallized from absolute alcohol, was obtained in 60% yield and an $[\alpha]^{25}D$ +8.0° (*c* 4% in water) and +30.5° (*c* 2% in 5 *N* HCl).

Anal. Calcd. for $C_5H_{11}O_3N$: C, 45.1; H, 8.2; N, 10.6. Found: C, 45.0; H, 8.3; N, 10.6.

The filtrate from the above, which contained the soluble lithium salt of formyl-O-methyl-D-allothreonine, was taken to dryness *in vacuo*. The residue was taken up in water and the lithium ion removed by passage of this solution through a column of 20 to 50 mesh Dowex 50 resin in the acid phase, as described previously.[29] Elution with water was continued until the formyl derivative, which appeared in the effluent as was indicated by a decrease in *p*H from 7 to about 3, was completely eluted (as indicated by rise in *p*H from 3 to 7). The fraction containing the formyl derivative was taken to dryness at 40° *in vacuo* and the residue dissolved in 100 ml. of 2 *N* HCl. After refluxing for 0.5 hr., the solution was concentrated to dryness, water was added and the evaporation repeated. The residue was dissolved in a small amount of water, neutralized to *p*H 6 by the addition of LiOH and subsequently evaporated to dryness. Solution of the residue was effected in boiling alcohol. Upon partial concentration, the D-form of O-methylallothreonine crystallized out. Recrystallization was effected from absolute ethanol. The amino acid, obtained in 55% yield, gave an $[\alpha]^{25}D$ −7.7° (*c* 4% in water) and −29.8° (*c* 2% in 5 *N* HCl).

Anal. Calcd. for $C_5H_{11}O_3N$: C, 45.1; H, 8.2; N, 10.6. Found: C, 45.3; H, 8.2; N, 10.6.

III. Preparation of Peptide Derivatives. Carbobenzoxy Amino Acids.—One-tenth mole each of the L- and L-alloforms of isoleucine and threonine, as well as D-alloisoleucine, were coupled with carbobenzoxy chloride (0.12 mole) in 2 *N* NaOH *via* the usual Schotten–Baumann procedure. The alkaline solutions were extracted with ether and the aqueous fraction acidified with 5 *N* HCl to congo red. Extraction of the carbobenzoxy amino acids was effected several times

(29) C. G. Baker, S-C. J. Fu, S. M. Birnbaum, H. A. Sober and J. P. Greenstein, THIS JOURNAL, **74**, 4701 (1952).

(30) K. Pfister, 3rd, E. E. Howe, C. A. Robinson, A. C. Shabica, E. W. Pietrusza and M. Tishler, *ibid.*, **71**, 1096 (1949).

(31) R. L. M. Synge, *Biochem. J.*, **33**, 1931 (1939).

with ethyl acetate and the combined extracts subsequently dried over anhydrous sodium sulfate. The filtrates were concentrated *in vacuo* to oily residues. Only the carbobenzoxy derivatives of L- and D-alloisoleucine could be crystallized. Recrystallization was effected from ethyl acetate by the addition of petroleum ether.

Carbobenzoxy-L-alloisoleucine: yield 76%, m.p. 65°, $[\alpha]^{27}D$ +16° (2% in acetone). *Anal.* Calcd. for $C_{14}H_{19}O_4N$: C, 63.3; H, 7.2; N, 5.3. Found: C, 63.2; H, 7.3; N, 5.2.

Carbobenzoxy-D-alloisoleucine: yield 73%, m.p. 65°, $[\alpha]^{27}D$ −15.8° (2% in acetone). *Anal.* Calcd. for $C_{14}H_{19}O_4N$: C, 63.3; H, 7.2; N, 5.3. Found: C, 63.3; H, 7.2; N, 5.3.

Carbobenzoxy-L-isoleucine: yield 85% (oil).

Carbobenzoxy-L-threonine: yield 60% (oil).

Carbobenzoxy-L-allothreonine: yield 66% (oil).

D-Alanine Benzyl Ester *p*-Toluenesulfonate.—The procedure employed was essentially that of Miller and Waelsch[32] with slight modification. A solution of 44.6 g. (0.5 mole) of D-alanine and 105 g. of *p*-toluenesulfonic acid monohydrate in 300 ml. of benzyl alcohol was distilled *in vacuo* at an oil-bath temperature of 120–130°. The distillation was repeated twice, subsequent to the addition each time of 300 ml. of benzyl alcohol. Addition of ether to the oily residue which remained caused it to solidify within a few minutes. The residue was dissolved in 180 ml. of absolute alcohol and precipitated by the addition of 1.2 l. of ether. The crystals were filtered over suction and dried at 50°, yield 133 g. (76%), m.p. 113–114°, $[\alpha]^{27}D$ +6.9° (2% in water).

Anal. Calcd. for $C_{17}H_{21}O_5NS$: C, 58.1; H, 6.0; N, 4.0. Found: C, 58.1; H, 6.1; N, 4.0.

Carbobenzoxy-L-isoleucyl-D-alanine Benzyl Ester.—The general procedure of Vaughan and Osato[19] was employed. A solution of 13.3 g. (0.05 mole) of carbobenzoxy-L-isoleucine and 7.0 ml. of triethylamine in 100 ml. of toluene was chilled to −5° and treated with 6.6 ml. of isobutyl chlorocarbonate. After 10 minutes, a cold solution of 17.6 g. of D-alanine benzyl ester *p*-toluenesulfonate and 7.0 ml. of triethylamine in 100 ml. of dichloromethane was added and the mixture allowed to stand overnight at 25°. A mass of crystals appeared which were filtered off after the addition of 200 ml. of petroleum ether. These were washed successively with water, dilute HCl, bicarbonate solution and water and then dried in air. Recrystallization from ethyl acetate gave a 16.2 g. (76%) yield of crystals with a m.p. of 171° and an $[\alpha]^{27}D$ −0.7° (2% in glacial acetic acid).

Anal. Calcd. for $C_{24}H_{30}O_5N_2$: C, 67.5; H, 7.1; N, 6.6. Found: C, 67.8; H, 7.0; N, 6.6.

Carbobenzoxy-L-alloisoleucyl-D-alanine Benzyl Ester.—The same procedure was used as for the preparation of the corresponding L-isoleucyl derivative described above; yield 15.3 g. (72%), m.p. 149°, $[\alpha]^{27}D$ +4.6° (2% in glacial acetic acid).

Anal. Calcd. for $C_{24}H_{30}O_5N_2$: C, 67.5; H, 7.1; N, 6.6. Found: C, 67.6; H, 6.9; N, 6.6.

Carbobenzoxy-D-alloisoleucyl-D-alanine Benzyl Ester.—The procedure for the coupling was essentially that given for the corresponding L-isoleucyl derivative above. However, after standing at 25° overnight, an additional 200 ml. of dichloromethane was added to the reaction mixture which was subsequently washed with water, dilute HCl, bicarbonate solution and water. The dichloromethane fraction was then dried over anhydrous sodium sulfate, the filtrate evaporated and the residual crystals recrystallized from ethyl acetate by the addition of petroleum ether; yield 17.1 g. (80%), m.p. 162°, $[\alpha]^{27}D$ +36.0° (2% in glacial acetic acid).

Anal. Calcd. for $C_{24}H_{30}O_5N_2$: C, 67.5; H, 7.1; N, 6.6. Found: C, 67.4; H, 7.2; N, 6.6.

The foregoing compound was also prepared *via* the procedure of Sheehan and Hess.[20] To a solution of 1.33 g. of carbobenzoxy-D-alloisoleucine, 1.76 g. of D-alanine benzyl ester *p*-toluenesulfonate and 0.7 ml. of triethylamine in 20 ml. of dichloromethane was added 1.09 g. of dicyclohexylcarbodiimide. The mixture was allowed to stand 4 hr. at 25°, and 0.05 ml. of acetic acid was then added. The precipitated dicyclohexylurea was removed, and the filtrate was washed with water, dilute HCl, bicarbonate solution, water and dried over sodium sulfate. The filtrate was treated in the same manner as above. The yield was 1.64 g. (78%), m.p. 162°, $[\alpha]^{27}D$ +35.8° (2% in glacial acetic acid).

Carbobenzoxy-L-threonyl-D-alanine Benzyl Ester.—To a solution of 12.7 g. of carbobenzoxy-L-threonine, 17.6 g. of D-alanine benzyl ester *p*-toluenesulfonate and 7.0 ml. of triethylamine in 200 ml. of dichloromethane was added 10.9 g. of dicyclohexylcarbodiimide.[20] The mixture was allowed to stand 4 hr. at 25° and 0.5 ml. of acetic acid was added. The precipitate was removed and the filtrate washed with water, dilute HCl, bicarbonate solution, water and dried over sodium sulfate. The filtrate was evaporated, and there remained a mixture of crystals and oil. This was treated with a small amount of dichloromethane and the insoluble dicyclohexylurea removed *via* filtration. The filtrate was evaporated and the residual oil converted to crystals upon drying in a vacuum desiccator over phosphorus pentoxide. These were filtered off with the aid of a small amount of ether (at this stage large amounts of crystals were lost, but when the residual material was recrystallized from ether and petroleum ether, or acetone and petroleum ether, the crystalline precipitate could not be filtered off due to its gelatinous nature). The white crystals were recrystallized from acetone by the addition of petroleum ether; yield 5.4 g. (26%), m.p. 147°, $[\alpha]^{27}D$ −0.6° (2% in glacial acetic acid).

Anal. Calcd. for $C_{23}H_{26}O_5N_2$: C, 63.7; H, 6.3; N, 6.8. Found: C, 63.7; H, 6.3; N, 6.9.

Carbobenzoxy-L-allothreonyl-D-alanine Benzyl Ester.—The same procedure was employed as for the corresponding L-threonine derivative described above. However, due to its lesser solubility in ether, this compound was more readily crystallized than the aforementioned; yield 7.6 g. (42%), m.p. 142–143°, $[\alpha]^{27}D$ +8.3° (2% in glacial acetic acid).

Anal. Calcd. for $C_{23}H_{26}O_5N_2$: C, 63.7; H, 6.3; N, 6.8. Found: C, 63.6; H, 6.3; N, 6.8.

L-Isoleucyl-D-alanine.—A suspension of 4.27 g. of carbobenzoxy-L-isoleucyl-D-alanine benzyl ester was treated with hydrogen in the presence of palladium black catalyst in the usual manner. At the termination of the reaction, the filtrate was evaporated *in vacuo* and the evaporation repeated three times subsequent to the addition, each time, of water. The residual crystals were filtered off with the aid of alcohol and recrystallized from water–alcohol. Physical constants, yields and analyses are given in Table VI.

Other Peptide Derivatives.—The L-alloisoleucyl-, D-alloisoleucyl-, L-threonyl- and L-allothreonyl derivatives of D-alanine were prepared in a manner analogous to that of L-isoleucyl-D-alanine described above. The pertinent data are presented in Table VI.

IV. Preparation of α-Hydroxy Acids. L-α-Hydroxy-β-methylvaleric Acid.—Six and one-half grams of L-isoleucine was dissolved in a mixture of 50 ml. of 1 *N* HCl, 200 ml. of water and 100 ml. of glacial acetic acid contained in a 1-l. beaker. The solution was chilled to about 5° in an ice-bath and a solution of 35 g. of sodium nitrite in 60 ml. of water added slowly, with continuous vigorous stirring and chilling (addition requires about 15 minutes). After the addition, the reaction mixture was allowed to stand at 5° for 1 hr. and at room temperature overnight. A Van Slyke manometric ninhydrin–CO_2 analysis of a 1-ml. aliquot showed the virtual absence of α-amino nitrogen after this time. After the addition of 50 ml. of concentrated HCl, the solution was evaporated to dryness *in vacuo* at 55°. Water was added and the evaporation repeated. The yellowish-brown residue was extracted several times with boiling acetone and the remaining white residue discarded. The combined acetone extracts were blown down to dryness in a jet of air, and the residue taken up in excess ether. After the ethereal extract had been dried with anhydrous sodium sulfate, it was concentrated to dryness. The crystalline residue was taken up in 20 ml. of boiling water and the solution decolorized with Norit. Concentrated sodium hydroxide solution was added dropwise to the cooled solution to *p*H 4.5 to 5. Addition of about three volumes of acetone to this solution led to the precipitation of the sodium salt of the hydroxy acid as beautiful gleaming prisms. These were filtered over suction, washed with acetone and dried *in vacuo;* yield 30–40%, $[\alpha]^{25}D$ −11.8° (4.62% in water).

Anal. Calcd. for $C_5H_{11}O_3Na$: C, 46.8; H, 7.1; Na, 14.9. Found: C, 46.9; H, 7.2; Na, 14.9.

Other α-Hydroxy Acids.—The α-hydroxy acids corresponding to D-isoleucine, L- and D-alloisoleucine, L- and D-

(32) H. K. Miller and H. Waelsch, THIS JOURNAL, **74**, 1092 (1952).

TABLE VI

PHYSICAL CONSTANTS, YIELDS AND ANALYSES OF NEW PEPTIDES

Dipeptide	Yield, %	Dec. point, °C.	$[\alpha]^{27}D^{a}$	Calculated, % C	Calculated, % H	Calculated, % N	Found, % C	Found, % H	Found, % N
L-Isoleucyl-D-alanine	93	259–260	+81.5°	53.4	9.0	13.9	53.4	8.9	13.9
L-Alloisoleucyl-D-alanine	85	245–247	+92.0	53.4	9.0	13.9	53.5	8.9	14.0
D-Alloisoleucyl-D-alanine	77	247–248	−23.9	53.4	9.0	13.9	53.2	9.0	13.8
L-Threonyl-D-alanine	67	139–142	+95.3	44.2	7.4	14.7	44.3	7.4	14.5
L-Allothreonyl-D-alanine	86	225–228	+87.5	44.2	7.4	14.7	43.9	7.5	14.7

[a] Concentration equals 2% in water.

TABLE VII

SPECIFIC ROTATIONS[a] OF α-HYDROXY ACIDS IN WATER AS THEIR SALTS AND CORRESPONDING FREE ACIDS

α-Hydroxy acid	Parent amino acid	$[\alpha]_D$ of α-hydroxy acid as Salt	$[\alpha]_D$ of α-hydroxy acid as Free acid[b]	Literature value $[\alpha]_D$
L-α-Hydroxy-β-methylvaleric (Na)	L-Isoleucine	−11.8°	+ 3.9°	+102°[c]
D-α-Hydroxy-β-methylvaleric (Na)	D-Isoleucine	+11.4	− 4.1	.....
L-*allo*-α-Hydroxy-β-methylvaleric (Na)	L-Alloisoleucine	− 5.2	+ 9.5	.....
D-*allo*-α-Hydroxy-β-methylvaleric (Na)	D-Alloisoleucine	+ 4.8	− 9.9	.....
L-Lactic (Zn)	L-Alanine	− 9.8	0	− 8.0 (as Zn salt)[d]
L-α-Hydroxybutyric (½Ba)	L-Butyrine	− 9.1	− 6.8	− 8.6 (as Ba salt)[e]
D-α-Hydroxybutyric (½Ba)	D-Butyrine	+ 8.8	+ 6.0	+ 8.6 (as Ba salt)[e]
L-α-Hydroxyvaleric (½Ba)	L-Norvaline	− 7.0	− 2.7	− 6.0 (as Ba salt)[e]
D-α-Hydroxyvaleric (½Ba)	D-Norvaline	+ 7.2	+ 2.2	+ 6.4 (as Ba salt)[e]
L-α-Hydroxycaproic (Na)	L-Norleucine	−15.9	− 4.2	− 9.3 (as Ba salt)[e]
D-α-Hydroxycaproic (Na)	D-Norleucine	+15.5	+ 3.8	+ 9.5 (as Ba salt)[e]
L-α-Hydroxyisovaleric (Na)	L-Valine	−15.2	− 1.0	−10.1 (as Ba salt)[e]
D-α-Hydroxyisovaleric (Na)	D-Valine	+15.2	+ 1.0	+10.0 (as Ba salt)[e]
L-α-Hydroxyisocaproic (Na)	L-Leucine	−22.8	−13.3	−27.7 (1% in *N* NaOH)[f]
D-α-Hydroxyisocaproic (Na)	D-Leucine	+22.8	+13.3	+19.4 (as Ba salt)[e]
L-α-Hydroxy-β-phenylpropionic	L-Phenylalanine	−54.2	−20.0	−13.3[g]
D-α-Hydroxy-β-phenylpropionic	D-Phenylalanine	+54.4	+20.4	.....
L-Malic (Ba)	L-Aspartic acid	0	0[h]	.....
D-Malic (Ba)	D-Aspartic acid	0	0	.....
L-α-Hydroxyglutaric (Ba)	L-Glutamic acid	− 5.3	0	− 1.98[i]
D-α-Hydroxyglutaric (Ba)	D-Glutamic acid	+ 5.1	0	+ 1.76[i]
L-Isocitric (tri-Na)[k]	L-Aminotricarballylic acid	0	+30.6	.....
D-Isocitric (tri-Na)[k]	D-Aminotricarballylic acid	0	−30.0	.....
L-Alloisocitric (tri-Na)[k]	L-Alloaminotricarballylic acid	+38.4	+34.6	.....
D-Alloisocitric (tri-Na)[k]	D-Alloaminotricarballylic acid	−38.8	−34.1	.....

[a] One to five per cent. solutions in a 2-dm. tube at 25–27°. [b] Obtained by neutralizing the corresponding salt with the equivalent amount of hydrochloric acid. In the case of the α-hydroxy-β-phenylpropionic acids, one equivalent of sodium hydroxide was used to secure the sodium salt. [c] As molybdate complex in water; O. Lutz and B. Jirgensons (*Ber.*, **65**, 784 (1932)). [d] E. Fischer and A. Skita (*Z. physiol. Chem.*, **33**, 177 (1901)); B. Iselin and E. Zeller (*Helv. Chim. Acta*, **29**, 1508 (1946)) and C. G. Baker and A. Meister (THIS JOURNAL, **73**, 1336 (1951)) reported values of −8.4 and −8.2°, respectively. [e] C. G. Baker and A. Meister. [f] H. Scheibler and A. S. Wheeler (*Ber.*, **44**, 2684 (1911)). Iselin and Zeller, and Baker and Meister reported values of −27.2 and −28.2°, respectively, in the same solvent. [g] Free acid in water; H. D. Dakin and H. W. Dudley (*J. Biol. Chem.*, **18**, 29 (1914)). [h] B. Holmberg (*Ber.*, **61**, 1893 (1928)) and Iselin and Zeller. [i] Free acid in water; E. O. von Lipmann, *Ber.*, **15**, 1156 (1882). [j] Free acid in water; E. O. von Lipmann, *Ber.*, **24**, 3301 (1891). [k] J. P. Greenstein, N. Izumiya, M. Winitz and S. M. Birnbaum, THIS JOURNAL, **77**, 707 (1955).

butyrine, L- and D-norvaline, L- and D-norleucine, L- and D-valine, L- and D-leucine, L- and D-phenylalanine, L- and D-aspartic acid and L- and D-glutamic acid were prepared by nitrous acid deamination of the optically pure[12] parent amino acids by the same procedure described above for L-α-hydroxy-β-methylvaleric acid. The hydroxy acids were isolated as their respective sodium salts with the exceptions of the α-hydroxybutyric, α-hydroxyvaleric, malic and α-hydroxyglutaric acids, obtained as their barium salts and the α-hydroxy-β-phenylpropionic acids, secured as the free acids. Optical rotation values for these compounds, both as the salt and as the free acid, are shown in Table VII.[33]

Preparation of α-Hydroxy Acid Oxidases.—In the present study, freshly secured rat kidney was homogenized with two volumes of a cold solution of 0.13 *M* potassium chloride in 0.13 *M* sodium phosphate buffer at *p*H 7.6. After the homogenate was strained through cheesecloth and another two volumes of buffer added thereto, the entire homogenate was centrifuged at 4° and 30,000 r.p.m. for 0.5 hr. The supernatant fraction was obtained by decantation, dialyzed against cold running tap water and subsequently lyophilized. The particulate fraction was washed with the same volume of buffer and obtained *via* centrifugation, followed by decantation of the wash fluid, which was discarded. Finally the particulates were suspended in 0.067 *M* phosphate buffer (*p*H 7.2), the suspension was heated at 60° for 0.5 hr.,

(33) It should be noted that the rule of Clough (*J. Chem. Soc.*, **107**, 1509 (1915); **113**, 526 (1918)) which states that all α-hydroxy acids showing parallel shifts in optical rotation are configurationally related, here applies. Thus an α-hydroxy acid of the L-configuration becomes more dextrorotatory on the addition of acid to its metal salt. This phenomenon, which obtains for α-amino (O. Lutz and B. Jirgensons, *Ber.*, **63**, 448 (1930); **64**, 1221 (1931); **65**, 784 (1932)), as well as α-hydroxy acids, is apparently associated with ionization of the carboxyl group. Examination of Table VII clearly reveals the applicability of this relationship to the monoasymmetric α-hydroxy acids whereas its application to the more complex α-amino and α-hydroxy acids was described in an earlier report (M. Winitz, S. M. Birnbaum and J. P. Greenstein, THIS JOURNAL, **77**, 716 (1955)).

dialyzed overnight in the cold against running tap water and lyophilized. The four diastereomers of α-hydroxy-β-methylvaleric acid were then subjected to the oxidative action of the supernatant and particulate fractions, so obtained.

Part of the enzymic data was obtained by Mrs. Betty Whitaker. The analyses were provided by Mr. Robert J. Koegel and his staff.

BETHESDA, MARYLAND

[CONTRIBUTION NO. 2053 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Chromatographic Separation and Identification of Some Peptides in Partial Hydrolysates of Tussah Silk Fibroin

BY LOIS M. KAY, W. A. SCHROEDER, NANCY MUNGER AND NATALIE BURT

RECEIVED NOVEMBER 21, 1955

From a partial hydrolysate of Tussah silk fibroin, 27 peptides have been isolated and identified. About two-thirds of the protein molecule has been accounted for by these peptides and by free amino acids in the hydrolysate. The structure and quantity of the peptides lead to the conclusion that repetition of adjacent alanine residues is an important feature in Tussah silk fibroin but that there is little regularity in the arrangement of other amino acids. This structure is in contrast to that of *Bombyx* silk fibroin in which, for the most part, glycine residues occupy alternate positions along the polypeptide chain.

That the structures of *Bombyx mori* silk fibroin (BSF) and of Tussah silk fibroin (TSF) differ has long been known from the X-ray diffraction investigations of Trogus and Hess.[1] Although differences in the structures might arise simply from varied arrangements in the spatial configuration of polypeptide chains of identical sequence, complete amino acid analyses of these fibroins[2] have shown that the differences in structure more probably result from the very definite dissimilarities of amino acid composition; in both fibroins the quantities of glycine and alanine account for about 75% of the residues, but the major difference lies in the almost exact reversal in the relative proportions of these two amino acids in one fibroin as compared to the other. Marsh, Corey and Pauling[3,4] have interpreted the X-ray diffraction patterns of the two fibroins in terms of antiparallel chain pleated sheets. Their structure for BSF indicates that glycine residues alternate in position along the polypeptide chains for the most part; chemical data[5] on the sequence of amino acids confirm this view. In TSF, the alanine content is such that, for the most part, alanine residues could occupy alternate positions along the chains; the X-ray data, however, cannot distinguish between this possible regular arrangement or a more random one.

Many years ago, Abderhalden and co-workers[6,7] isolated alanine anhydride and what was thought to be ala-ala-ala-gly[8] from TSF. If these identifications are correct an alternate arrangement of alanine residues seems unlikely. The results of our investigation confirm this conclusion. In the present paper we shall describe the isolation and identification of 27 peptides in a partial hydrolysate of Tussah silk fibroin.

(1) C. Trogus and K. Hess, *Biochem. Z.*, **260**, 376 (1933). See also W. T. Astbury and F. O. Bell, *Tabulae Biologicae*, **17**, 90 (1939), for a résumé.

(2) W. A. Schroeder, L. M. Kay, B. Lewis and N. Munger, THIS JOURNAL, **77**, 3908 (1955).

(3) R. E. Marsh, R. B. Corey and L. Pauling, *Biochim. Biophys. Acta*, **16**, 1 (1955).

(4) R. E. Marsh, R. B. Corey and L. Pauling, *Acta Cryst.*, **8**, 710 (1955).

(5) L. M. Kay and W. A. Schroeder, THIS JOURNAL, **76**, 3564 (1954), and unpublished data.

(6) E. Abderhalden and A. Suwa, *Z. physiol. Chem.*, **66**, 13 (1910).

(7) E. Abderhalden and K. Heyns, *ibid.*, **202**, 37 (1931).

(8) Abbreviation of amino acid names and representation of sequences follow E. Brand (*Ann. N. Y. Acad. Sci.*, **47**, 187 (1946)) and F. Sanger (*Advances in Protein Chem.*, **7**, 1 (1952)).

Experimental

Source and Partial Hydrolysis of TSF.—The TSF which was used for hydrolysis was a portion of the preparation which had been completely analyzed in previous work.[2]

A 1.07-g. sample of TSF was placed in a 25-ml. volumetric flask and 10 ml. of J. T. Baker analyzed concd. hydrochloric acid was added. The mixture was maintained at 37° for 48 hr. with constant agitation which was supplied by attaching the stem of the flask parallel to a shaft which was inclined at 45° and rotated at 25 r.p.m. The greater portion of the fibroin dissolved rapidly to form a dark orange-brown solution which in the course of hydrolysis became darker and took on a greenish tinge. At the end of the period of hydrolysis, the mixture was cooled to room temperature, diluted to 25 ml. with water and a portion was used for the chromatogram. The solution contained suspended, undissolved material which was investigated as described below.

Ion-exchange Chromatography of the Partial Hydrolysate.—Chromatography was carried out on a column of Dowex 50-X4, 3.5 × 100 cm. in dimension. The resin (200–400 mesh, high porosity, Lot No. 3198-42) was purified exactly as Moore and Stein[9] describe for Dowex 50-X8 and was passed through a 120-mesh sieve.[10] The column was packed in the manner which Moore and Stein suggest. In the preparation of buffers, the detergent BRIJ 35, benzyl alcohol, thiodiglycol and disodium Versenate were omitted; *p*H 3.42 buffer was prepared as they describe and *p*H 5.5 (±0.05) buffer by minor modification of the quantities required for *p*H 5.0 buffer for basic amino acids.

The sample to be chromatographed was made up in the following way. A 13-ml. portion of diluted hydrolysate (preceding section) was pipetted into a mixture of 40 ml. of *p*H 6.7 buffer, 9 ml. of 6 *N* sodium hydroxide and 1 ml. of water. The *p*H of the solution was adjusted to 2.3 with 0.5 ml. of 6 *N* hydrochloric acid. After centrifuging to remove suspended material, 60 ml. of solution was placed on the column. On a moisture- and ash-free basis, the sample contained the soluble portion from 488 mg. of TSF.

Throughout the chromatogram, the temperature was maintained at 37°. Development was begun with *p*H 3.42 buffer and at the point shown in Fig. 1, change to *p*H 5.5

(9) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

(10) More recently it has been determined that about one-third of the material in this sample of resin will pass a 200-mesh sieve. Consequently, the column as used contained a mixture of one-third through-200-mesh resin and two-thirds 120–200 mesh resin.