[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY OF BAYLOR UNIVERSITY COLLEGE OF MEDICINE]

Stereoisomerism of N,N'-Oxalylbis-(alanine) Derivatives[1]

BY WALTER R. HEARN AND RICHARD A. HENDRY

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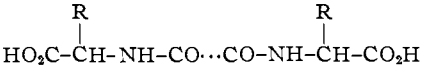
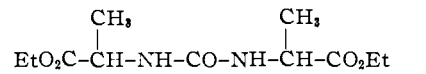
The four stereoisomers of N,N'-oxalylbis-(alanine) and its methyl and ethyl esters were prepared. Papain-catalyzed anilide synthesis was used to characterize the four isomers: the L-form and racemic form yielded a precipitate of N,N'-oxalylbis-(L-alanine anilide); the D-form and *meso* form did not yield a precipitate under the same reaction conditions.

A number of N,N'-diacyl derivatives of α-amino acids have been described in the literature. When such compounds are prepared from racemic amino acids or their derivatives by reaction with a symmetrical acylating agent, the product will contain two identical asymmetric carbon atoms and should be capable of existing in both racemic and *meso* forms.

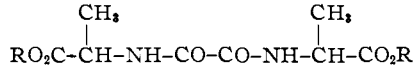


Only one paper describing the characterization of the racemic and *meso* forms of a diacylbis-(α-amino acid) derivative has come to our attention. Gränacher prepared carbonylbis-(alanine ethyl ester) by the action of phosgene on DL-alanine ethyl ester[2] and later fractionated the crude product into two isomers, m.p. 85 and 153°, respectively.[3] Both esters on saponification in ethanolic potassium hy-



droxide yielded the same acid, resolvable by the use of strychnine. Since this racemic acid gave the 153°-melting ester on treatment with diazoethane, the high-melting ester was regarded as the racemic form, and the low-melting ester as the *meso* form; inversion was presumed to have occurred when the *meso* ester was treated with the alcoholic base.

In connection with another problem, we became interested in oxalyl derivatives of amino acids. Oxalylbis-(α-amino acid) derivatives have been prepared by a number of investigators,[4-9] and in several cases in which racemic amino acids were used as starting materials, isomeric products have been reported. Thus, Schiff[4] refluxed DL-alanine in diethyl oxalate containing 5–10% ethanol and obtained a small amount of oxalylbis-(alanine ethyl ester) in addition to oxalylbis-(alanine), the principal product; after tedious fractional crystallization, the ester was separated into two isomers, m.p. 125–127° and 152–154°, respectively. The crude acid could not be crystallized, but on treatment with ethanolic hydrogen chloride yielded a mixture of the above esters which could again be separated by fractional crystallization. Bornwater[6] prepared oxalylbis-(alanine methyl ester) from DL-alanine methyl ester hydrochloride by reaction with oxalyl chloride in refluxing benzene, and by fractional crystallization was able to separate the product into two isomers, m.p. 120–121° and 155–156°, respectively. Although our melting points do not agree exactly with those obtained by Schiff and Bornwater, we have succeeded in characterizing the stereoisomers of N,N'-oxalylbis-(alanine ethyl ester) (I), and N,N'-oxalylbis-(alanine methyl ester) (II).



I, R = Et; II, R = Me

In the course of this work, various methods of synthesis of oxalylbis-(amino acid) derivatives were compared. Kerp and Unger[5] prepared oxalylbis-(glycine) in unspecified yield by the reaction of diethyl oxalate and glycine in aqueous potassium hydroxide; by a slight modification of their procedure our yield was increased to a maximum of 38%. Failure of this reaction with other amino acids was confirmed. Reaction of oxalyl chloride with two moles of the amino acid ester hydrochloride suspended in refluxing benzene[6] was found to be generally satisfactory. Occasionally the use of another solvent improved the yield. Substituting ethyl acetate for benzene increased the yield of oxalylbis-(L-tyrosine ethyl ester) from 10% of theoretical to 53%, but with other amino acids yields of greater than 70% were usually obtained. We found, in agreement with Cleaver and Pratt,[10] that the reaction of an amino acid with oxalyl chloride in anhydrous pyridine, ether or dioxane gave low yields of the desired product; but in contrast to their findings, we did not experience losses in the hydrolysis of oxalylbis-(amino acid esters) to the free acids. Saponifications were carried out at room temperature using a twofold excess of decinormal to normal aqueous sodium hydroxide, generally for one hour. Yields of the free acid were usually greater than 80% after neutralization with hydrochloric acid or a cation-exchange resin in the hydrogen phase.

Oxalylbis-(alanine methyl ester) and oxalylbis-(alanine ethyl ester) were prepared by the action of oxalyl chloride on DL-alanine methyl ester hydrochloride and DL-alanine ethyl ester hydrochloride, respectively. Fractional crystallization of the

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(2) C. Gränacher and H. Landolt, *Helv. Chim. Acta*, **10**, 799 (1927).

(3) C. Gränacher and G. Wolf, *ibid.*, **11**, 172 (1928).

(4) H. Schiff, *Ber.*, **18**, 490 (1885); **17**, 401, 1033 (1884).

(5) W. Kerp and K. Unger, *ibid.*, **30**, 579 (1897).

(6) J. T. Bornwater, *Rec. trav. chim.*, **31**, 105 (1912).

(7) J. T. Bornwater, *ibid.*, **35**, 124 (1916); **36**, 250 (1916).

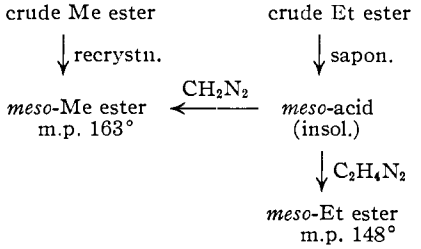
(8) D. J. Meijeringh, *ibid.*, **32**, 140 (1913).

(9) E. Abderhalden, E. Rindtorff and A. Schmitz, *Fermentforschung*, **10**, 213 (1928).

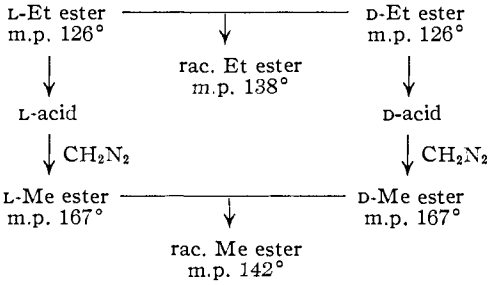
(10) C. S. Cleaver and B. C. Pratt, THIS JOURNAL, **77**, 1544 (1955).

crude methyl ester yielded a single isomeric modification, m.p. 163°, but repeated recrystallization of the crude ethyl ester failed to yield a modification with a sharp melting point. To obtain one of the isomers of the ethyl ester, the racemic modification was prepared by mixing equimolar amounts of the D- and L-esters, synthesized from D- and L-alanine ethyl ester hydrochloride, respectively. This racemic N,N′-oxalylbis-(alanine ethyl ester) melted sharply at 138°.

Saponification of the crude oxalylbis-(alanine ethyl ester) was carried out, and on acidification approximately 35% of the theoretical amount of oxalylbis-(alanine) precipitated. This insoluble acid was collected by filtration and recrystallized several times. Treatment with diazomethane gave the same oxalylbis-(alanine methyl ester), m.p. 163°, that had been crystallized previously from the product of oxalyl chloride on DL-alanine methyl ester hydrochloride. Treatment with diazoethane, however, gave a new oxalylbis-(alanine ethyl ester), m.p. 147–148°, different from the synthetic racemate previously prepared and therefore the *meso* isomer. The insoluble acid was therefore *meso*-oxalylbis-(alanine).



Subsequently, the D- and L-ethyl esters were hydrolyzed to the D- and L-acids, and the acids treated individually with diazomethane to yield the D- and L-methyl esters. The racemic N,N′-oxalylbis-(alanine methyl ester) prepared by mixing equimolar amounts of these two derivatives melted at 141–142°.



The racemic acid prepared by mixing equimolar amounts of the L-acid and D-acid, or by saponification of the racemic esters, proved to be much more soluble in water than the *meso*-acid, as was expected from the previous behavior of the crude mixture of acids. The decomposition points were 195–205° for the L- and D-acids, 235–240° for the racemic form and 275° for the *meso*-acid. The properties of the esters are summarized in Table I.

Enzyme Experiments.—For confirmation of the characterization of the racemic and *meso*-acids we turned to stereospecific enzymatic reactions. The separation of the racemic and *meso*-forms of

TABLE I

ESTERS OF N,N′-OXALYLBIS-(ALANINE)

Stereoisomeric form	M.p., °C.	$[\alpha]^{25}D$
N,N′-Oxalylbis-(L-alanine methyl ester)	167	−69.5°[a]
N,N′-Oxalylbis-(D-alanine methyl ester)	167	+70.2°[a]
rac.-N,N′-Oxalylbis-(alanine methyl ester)	141–142	.....
meso-N,N′-Oxalylbis-(alanine methyl ester)	163	.....
N,N′-Oxalylbis-(L-alanine ethyl ester)	126	−40.1[b]
N,N′-Oxalylbis-(D-alanine ethyl ester)	126	+44.9[b]
rac.-N,N′-Oxalylbis-(alanine ethyl ester)	138	.....
meso-N,N′-Oxalylbis-(alanine ethyl ester)	147–148	.....

[a] (*c* 1.00, glacial acetic acid). [b] (*c* 1.00, 95% ethanol)

α,ε-diaminopimelic acid recently has been accomplished by the use of a renal amidase–Mn^{++} preparation to hydrolyze the diamide of the synthetic acid.[11] The products of the enzymatic hydrolysis were the L,L-diaminopimelic acid, the D,D-diamide and the L-diaminopimelic acid D-monoamide, which were separated on a cation-exchange resin and hydrolyzed separately to yield the L-, the D- and the *meso*-diaminopimelic acids, respectively. The insolubility of the amides prevented the use of an analogous reaction in our case.

We considered the possibility of using renal acylase I for the stereospecific cleavage of the oxalyl-L-alanine bond because of the reported rather broad specificity of this enzyme toward the acyl moiety of N-acylamino acids.[12] Oxalylbis-(glycine) and oxalylbis-(L-alanine) were subjected to the action of a renal acylase preparation, with acetyl-DL-alanine as a control substrate run under identical conditions. After 2 hours of incubation, the acetyl derivative (of the L-form) had been hydrolyzed to the extent of 96%, but there was no evidence of any action on either of the oxalyl derivatives.

The action of pancreatic carboxypeptidase on N-acylamino acids is similar to that of renal acylase I, except that carboxypeptidase is more effective toward derivatives of aromatic amino acids than toward derivatives of aliphatic amino acids, while the reverse is true for acylase.[12] It is interesting in this connection that we observed some hydrolysis of oxalylbis-(L-tyrosine) by carboxypeptidase (35% in 18 hours as compared with 98% in 1 hour for chloroacetyl-L-tyrosine, the control substrate). We did not attempt to use this enzyme to distinguish between oxalylbis-(alanine) isomers because we assumed that they would be hydrolyzed even more slowly if at all.

We finally investigated the stereospecific synthesis of anilides catalyzed by cysteine-activated papain.[12,13] In a preliminary experiment, N,N′-oxalylbis-(glycine) was found to give a 52% yield of N,N′-oxalylbis-(glycine anilide) after 96 hours of incubation with the enzyme and aniline in acetate buffer at *p*H 4.7 at 40°. It is interesting to note that N,N′-carbonylbis-(glycinamide) has been shown not to serve as a substrate for papain-catalyzed ammonia liberation.[14]

When oxalylbis-(L-alanine) was incubated with cysteine–papain and aniline under the same condi-

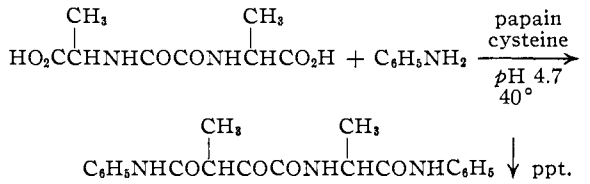
(11) E. Work, S. M. Birnbaum, M. Winitz and J. P. Greenstein, THIS JOURNAL, **77**, 1916 (1955).

(12) J. P. Greenstein, *Advances in Protein Chem.*, **9**, 121 (1954).

(13) M. Bergmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, **119**, 707 (1937).

(14) J. W. Clark-Lewis and J. S. Fruton, *ibid.*, **207**, 477 (1955).

tions used for the experiment with the glycine derivative, a precipitate of N,N'-oxalylbis-(L-alanine anilide) was obtained.



When N,N'-oxalylbis-(D-alanine) was incubated under the same conditions, no precipitate was obtained. This reaction was now carried out on the mixture of acids obtained by saponification of the crude oxalylbis-(alanine ethyl ester) from DL-alanine and also on the less soluble component of this mixture. A precipitate of N,N'-oxalylbis-(L-alanine anilide) was obtained from the crude mixture of racemic and *meso*-acids but not from the less soluble acid. Subsequent crops of more soluble acid from the mixture did yield the anilide on incubation with the enzyme and aniline. These enzymatic experiments therefore confirm that the less soluble oxalylbis-(alanine) is the *meso*-form (dec. 275°); any monoanilide formed from the action of the enzyme on the L-portion of this isomer remained in solution, subject to enzymatic hydrolysis back to the free acid. On the other hand, the L,L-portion of the racemic form (dec. 235–240°) reacted to form the highly insoluble bis-anilide, which immediately precipitated from the solution.[15]

Experimental[16]

N,N'-Oxalylbis-(glycine). Method A.—This compound was prepared using the procedure of Kerp and Unger.[5] Eighty grams (0.55 mole) of diethyl oxalate was added to a solution of 60 g. of potassium hydroxide and 82 g. (1.10 moles) of glycine in 750 ml. of water. The diethyl oxalate rapidly went into solution with the production of heat and the mixture was kept at 40° for two hours. The solution was acidified to *p*H 2 with concentrated hydrochloric acid and kept overnight in the refrigerator. The white, crystalline acid was filtered, washed with cold water, and dried. The product melted with decomposition at 250–255° (Kerp and Unger, m.p. 250°), 20 g. (17%).

Anal. Calcd. for $C_6H_8O_6N_2$: N, 13.7; neut. equiv., 102. Found: N, 13.4; neut. equiv., 104.

Method B.—When the above procedure was carried out using half the volume of water (375 ml.), acidification with hydrochloric acid yielded 56 g. (42%) of the monopotassium salt of N,N'-oxalylbis-(glycine). The salt decomposed, 235–240°, and was somewhat hygroscopic.

Anal. Calcd. for $C_6H_7O_6N_2K$: C, 29.8; H, 2.92; N, 11.6; neut. equiv., 242. Found: C, 29.5; H, 3.42; N, 11.32; neut. equiv., 240.

The free acid was prepared by dissolving 10 g. of the potassium salt in 100 ml. of boiling water and acidifying to *p*H 2 with hydrochloric acid. On cooling, 7.0 g. (84% based on salt, or 35% over-all) of N,N'-oxalylbis-(glycine) was obtained.

Anal. Calcd. for $C_6H_8O_6N_2$: neut. equiv., 102. Found: neut. equiv., 104.

Several modifications of the above conditions failed to yield a product when alanine was substituted for glycine.

Method C.—Five grams of N,N'-oxalylbis-(glycine ethyl ester) (see below) was dissolved with shaking over a period of 1.5 hours in 200 ml. of 0.2 *N* sodium hydroxide. The solution was filtered and the filtrate acidified to *p*H 2 with hydrochloric acid. After standing overnight in the refrigerator, 2.5 g. (65%) of the crystalline product was obtained. Concentration of the mother liquor yielded an additional 0.7 g., total 3.2 g. (83%).

Anal. Calcd. for $C_6H_8O_6N_2$: neut. equiv., 102. Found: neut. equiv., 102.

N,N'-Oxalylbis-(glycine ethyl ester) was prepared by the procedure of Bornwater.[6] To a suspension of 22 g. (0.16 mole) of glycine ethyl ester hydrochloride in 250 ml. of sodium-dried benzene was added 10 g. (0.08 mole) of oxalyl chloride. The mixture was refluxed under anhydrous conditions for four hours; by this time the ester hydrochloride had gone into solution and the evolution of hydrogen chloride had ceased. The benzene solution was decanted from slight traces of unreacted material and on cooling the product crystallized and was obtained by filtration. The product melted at 139° (Bornwater, m.p. 143°), 19 g. (93%). An additional crop of 1.5 g. of the ester was obtained on concentration of the filtrate to dryness *in vacuo* and recrystallization of the residue from water; total, 20.5 g. (99%).

Anal. Calcd. for $C_{10}H_{16}O_6N_2$: C, 46.2; H, 6.15; N, 10.8. Found: C, 46.2; H, 6.13; N, 11.1.

N,N'-Oxalylbis-(L-tyrosine ethyl ester) was prepared by the same general method described above for the glycine ethyl ester derivative, but the yield was only 10% with benzene as the solvent. In this case, ethyl acetate dried over calcium chloride gave better yields. Ten grams (0.04 mole) of L-tyrosine ethyl ester hydrochloride and 2.6 g. (0.02 mole) of oxalyl chloride were allowed to react as above in 125 ml. of ethyl acetate. Concentration of the ethyl acetate to half its volume gave 3.5 g. (37%) of crude product. Further concentration yielded an additional 1.5 g. Recrystallization several times from 1:1 alcohol–water gave a product, m.p. 197°, $[\alpha]^{25}D$ + 42.0° (*c* 1.0, 95% ethanol), 5.0 g. (53%).

Anal. Calcd. for $C_{24}H_{28}O_8N_2$: C, 61.0; H, 5.97; N, 5.93. Found: C, 60.87; H, 6.07; N, 6.06.

N,N'-Oxalylbis-(L-tyrosine).—One gram of N,N'-oxalylbis-(L-tyrosine ethyl ester) was treated with a solution of 35 ml. of 0.15 *N* sodium hydroxide and 10 ml. of ethanol. On acidification of the solution with hydrochloric acid to *p*H 2, the product precipitated, was collected by filtration, washed and dried. The yield was 0.7 g. (80%). After several recrystallizations from alcohol and water the compound melted with decomposition at 245–247°, $[\alpha]^{25}D$ +138.4° (*c* 1.1, 95% ethanol).

Anal. Calcd. for $C_{20}H_{20}O_8N_2$: C, 57.7; H, 4.84; N, 6.73; neut. equiv., 208. Found: C, 56.4; H, 5.05; N, 6.48; neut. equiv., 215.

Mixture of Isomers of N,N'-Oxalylbis-(*dl*-alanine methyl ester).[17]—A suspension of 16.0 g. (0.113 mole) of DL-alanine methyl ester hydrochloride in 150 ml. of dry benzene was refluxed with 7.3 g. (0.058 mole) of oxalyl chloride. Concentration of the benzene solution yielded 11.6 g. (78%) of the crude solid product, which after one recrystallization from water melted at 130–145°.

Anal. Calcd. for $C_{10}H_{16}O_6N_2$: C, 46.2; H, 6.19; N, 10.8. Found: C, 45.98; H, 6.15; N, 10.96.

Isolation of a Single Isomer of N,N'-Oxalybis-(*dl*-alanine methyl ester).—Sevenfold recrystallization of the above mixture from water yielded a single isomer, melting at 163° (Bornwater,[6] m.p. 155–156°, after three recrystallizations).

Mixture of Isomers of N,N'-Oxalylbis-(*dl*-alanine ethyl ester).—A suspension of 12.3 g. (0.08 mole) of DL-alanine ethyl ester hydrochloride in 125 ml. of dry benzene was refluxed for four hours with 5.0 g. (0.04 mole) of oxalyl chloride. Concentration of the benzene solution yielded 8.0 g. (71%) of product melting over a wide range, 120–130°.

(15) After preparation of this manuscript, our attention was called by a footnote in a paper by R. Wade, S. M. Birnbaum, M. Winitz, R. J. Koegel and J. P. Greenstein, THIS JOURNAL, **79**, 649 (1957), to the work of Y. Izumi, *J. Chem. Soc. Japan*, **75**, 1152 (1954). Izumi (*C. A.*, **49**, 11550*i* (1955)) also has reported the use of papain-catalyzed anilide synthesis to distinguish racemic and *meso* forms, in his case the isomers of dibenzoyl-α,ε-diaminopimelic acid.

(16) Melting points were determined on a Fisher–Johns melting point block and are uncorrected. Elemental analyses by Huffman Microanalytical Laboratories, Wheatridge, Colo. We are indebted to Miss Lilita Straumanis of the M. D. Anderson Hospital, Houston, for assistance wth the optical rotation measurements.

(17) Nomenclature of the mixtures of racemic and *meso* isomers is that used in ref. 10.

Repeated recrystallizations from water failed to yield a single modification of sharp m.p.

Anal. Calcd. for $C_{12}H_{20}O_6N_2$: C, 50.0; H, 6.99; N, 9.72. Found: C, 50.0; H, 6.94; N, 9.80.

N,N'-Oxalylbis-(D-alanine ethyl ester) was prepared by the general procedure described above from 12.8 g. (0.083 mole) of D-alanine ethyl ester hydrochloride; yield 10.3 g. (86%). Recrystallization from water gave needles, m.p. 126°, $[\alpha]^{25}D$ + 44.9° (*c* 1.00, 95% ethanol).

Anal. Calcd. for $C_{12}H_{20}O_6N_2$: C, 50.0; H, 6.99; N, 9.72. Found: C, 49.99; H, 6.87; N, 9.64.

N,N'-Oxalylbis-(L-alanine ethyl ester) was prepared in the same manner from 11 g. (0.07 mole) of L-alanine ethyl ester hydrochloride; yield 7.0 g. (68%). Recrystallization from water gave needles, m.p. 126°, $[\alpha]^{25}D$ −40.1° (*c* 1.00, 95% ethanol).

Anal. Calcd. for $C_{12}H_{20}O_6N_2$: C, 50.0; H, 6.99; N, 9.72. Found C, 50.0; H, 6.96; N, 9.80.

Racemic N,N'-Oxalylbis-(alanine ethyl ester) Prepared from the D and L-Isomers.—By addition of 25.0 mg. of N,N'-oxalylbis-(D-alanine ethyl ester) to 25.0 mg. of N,N'-oxalylbis-(L-alanine ethyl ester) the racemic modification was obtained. The racemate after several recrystallizations from water melted sharply at 138°.

Mixture of Isomers of N,N'-Oxalylbis-(*dl*-alanine).—Five grams of the mixture of isomers of N,N'-oxalylbis-(*dl*-alanine ethyl ester) was saponified in 100 ml. of 0.70 *N* NaOH at room temperature. On acidification with hydrochloric acid, 1.5 g. (38%) of product decomposing at 268° was obtained. Concentration of the mother liquor yielded subsequent crops of progressively lower decomposition points for a total yield of about 2.5 g. (63%).

Isolation of a Single Isomer of N,N'-Oxalylbis-(*dl*-alanine). ***meso*-N,N'-Oxalylbis-(alanine).**—Recrystallization from water of the first crop of crystals obtained in the above experiment yielded a crystalline product decomposing at 275°, shown to be the *meso* isomer by the following experiment.

Anal. Calcd. for $C_8H_{12}O_6N_2$: C, 41.4; H, 5.21; N, 12.1; neut. equiv., 116. Found: C, 41.4; H, 5.12; N, 12.18; neut. equiv., 118.

***meso*-N,N'-Oxalylbis-(alanine ethyl ester)** was prepared in quantitative yield by treatment of the above acid, dec. 275°, with diazoethane.[18] Recrystallization of the ester from water gave a product, m.p. 147–148°. Since this optically-inactive compound and the previously prepared racemic N,N'-oxalylbis-(alanine ethyl ester) are not identical, this must be the *meso* isomer and the acid from which it was prepared must be the *meso*-acid.

Anal. Calcd. for $C_{12}H_{20}O_6N_2$: C, 50.0; H, 6.99; N, 9.72. Found: C, 50.0; H, 7.14; N, 9.63.

***meso*-N,N'-Oxalylbis-(alanine methyl ester).**—Treatment of the *meso*-N,N'-oxalylbis-(alanine) with an excess of diazomethane in ether[19] gave a quantitative yield of this ester. Recrystallization from water gave a product, m.p. 163°. A mixed m.p. with the isomer previously isolated from the mixture of methyl esters showed no depression, indicating that this less soluble form was the *meso* isomer.

Anal. Calcd. for $C_{10}H_{16}O_6N_2$: C, 46.2; H, 6.19; N, 10.8. Found: C, 46.2; H, 6.18; N, 10.71.

N,N'-Oxalylbis-(D-alanine).—This acid and its enantiomorph were found to be very soluble in water and difficult to purify. To 50 ml. of 0.7 *N* NaOH (0.035 mole) was added 2.5 g. (0.0087 mole) of N,N'-oxalylbis-(D-alanine ethyl ester). The mixture was shaken at frequent intervals until all the ester had dissolved. The solution was then treated twice with 80–90 ml. of a slurry of Nalcite-HCR, 20–50 mesh, in the hydrogen form. After removal of the resin by filtration the filtrate was evaporated to dryness at 50° *in vacuo*. The acid melted with decomposition over a range 195–205°, 1.4 g. (70%).

Anal. Calcd. for $C_8H_{12}O_6N_2$: C, 41.4; H, 5.21; N, 12.1; neut. equiv., 116. Found: C, 40.1; H, 5.06; N, 11.83; neut. equiv., 123.

N,N'-Oxalylbis-(L-alanine) was prepared in a similar manner from N,N'-oxalylbis-(L-alanine ethyl ester), m.p. 195–205° dec. (yield 93%).

Anal. Calcd. for $C_8H_{12}O_6N_2$: C, 41.1; H, 5.21; N, 12.1; neut. equiv., 116. Found: C, 41.0; H, 5.14; N, 11.94; neut. equiv., 122.

N,N'-Oxalylbis-(D-alanine methyl ester) was prepared in quantitative yield by the action of diazomethane in ether on an ethanolic solution of N,N'-oxalylbis-(D-alanine). Recrystallization of the ester from water gave a product, m.p. 167°, $[\alpha]^{25}D$ +70.2° (*c* 1.00, glacial acetic acid).

Anal. Calcd. for $C_{10}H_{16}O_6N_2$: C, 46.2; H, 6.19; N, 10.80. Found: C, 46.2; H, 6.39; N, 10.84.

N,N'-Oxalylbis-(L-alanine methyl ester) was prepared in a similar manner from N,N'-oxalylbis-(L-alanine). The product was recrystallized from water, m.p. 167°, $[\alpha]^{25}D$ −69.5° (*c* 1.00, glacial acetic acid).

Anal. Calcd. for $C_{10}H_{16}O_6N_2$: C, 46.2; H, 6.19; N, 10.80. Found: C, 46.2; H, 6.08; N, 10.91.

Racemic N,N'-Oxalylbis-(alanine methyl ester) Prepared from the D- and L-Isomers.—This racemic modification was prepared by mixing 5.0 mg. of each of the two enantiomorphs and recrystallizing the mixture from water. The product melted at 141–142°.

Enzyme Experiments. Acylase.—The enzyme solution was prepared by dissolving 60 mg. of hog kidney acylase (Armour, tech. grade, assayed at 316 acylase units/g.) in 8 ml. of water and centrifuging to remove solid matter. The individual substrates were monopotassium oxalylbis-(glycinate) (0.0125 *M*), oxalylbis-(L-alanine) (0.0125 *M*) and acetyl-DL-alanine (0.025 *M*). To 1 ml. of each substrate were added 1 ml. of phosphate buffer (0.1 *M*, *p*H 7.2) and 1 ml. of enzyme solution; the tubes were then placed in an incubator set at 37°. A reagent blank was run simultaneously using 1 ml. of water in place of the substrate.

At the end of the incubation period 7 ml. of tungstic acid solution (prepared the same day by adding 10 ml. of 10% sodium tungstate to 80 ml. of 0.1 *N* sulfuric acid) was added to each tube and the precipitated protein removed by centrifugation. A sample of 4 ml. of each supernatant was transferred to a 10-ml. volumetric flask and made to volume with distilled water. The amount of free amino acid in 0.5 ml. of this solution was then determined by the colorimetric ninhydrin method of Troll and Cannan,[20] using DL-alanine solutions to plot the standard curve.

Under these conditions the control substrate, acetyl-DL-alanine, was hydrolyzed to the extent of 96% of the theoretical maximum in two hours and 100% in four hours, but neither of the two oxalyl derivatives was hydrolyzed to any measurable extent.

Carboxypeptidase.—The enzyme solution was prepared by the addition of 4 mg. of pancreatic carboxypeptidase (Nutritional Biochemicals Corp., crystallized 3×) to 5 ml. of 1% sodium bicarbonate. Substrates were oxalylbis-(L-tyrosine) (0.00625 *M*) and chloroacetyl-L-tyrosine (0.01 *M*).

To 1 ml. of water were added 1 ml. of substrate, 1 ml. of buffer (0.06 *M* veronal, 0.3 *M* sodium chloride, *p*H 7.5[21]) and 1 ml. of enzyme solution, and the tubes were placed in an incubator at 38–40°. At various time intervals, 0.3-ml. samples were removed and added to 0.7 ml. of tungstic acid solution (prepared as for the acylase experiments). Then another 1.5 ml. of water was added, the precipitated protein removed by centrifugation, and 0.5 ml. of the supernatant analyzed for free amino acid as in the acylase experiments.

The substrates were hydrolyzed to the following extent: chloroacetyl-L-tyrosine: 0.5 hr., 95%; 1 hr., 98%; 2 hr., 100%; oxalylbis-(L-tyrosine): 4 hr., 13.5%; 7 hr., 28%; 18 hr., 35.5%; 24 hr., 35.5%.

Papain. Preparation of N,N'-Oxalylbis-(glycine anilide).—To 250 ml. of 0.5 *M* acetate buffer, *p*H 4.7, were added 10.2 g. (0.05 mole) of N,N'-oxalylbis-(glycine), 1.2 g. of L-cysteine hydrochloride and 9.3 g. (0.10 mole) of redistilled aniline. To this solution was added 23 ml. of a papain extract, prepared by extracting 15 g. of papain (Nutritional Biochemicals Corp.) with 80 ml. of water for one hour at 5°, and centrifuging to remove insoluble matter. The reaction

(18) E. A. Werner, *J. Chem. Soc.*, **115**, 1093 (1919); F. Arndt and H. Scholz, *Angew. Chem.*, **46**, 47 (1933).

(19) F. Arndt, "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1943, pp. 165, 461.

(20) W. Troll and R. K. Cannan, *J. Biol. Chem.*, **200**, 803 (1953).

(21) H. Neurath, in S. P. Colowick and N. O. Kaplan, "Methods in Enzymology," vol. II, Academic Press, New York, N. Y., 1955, p. 79.

was carried out in tightly stoppered bottles containing very little free air space, in an incubator set at 40°. After 4 days the precipitate which formed was filtered and washed successively with water, 2% sodium bicarbonate, 0.1 *N* HCl, and again with water. The anilide was dissolved in boiling dimethylformamide (DMF), treated with Norit, and the solution filtered. On cooling, the anilide precipitated as a white powder which decomposed slightly but did not melt below 315°, yield 10.0 g. (52%).

Anal. Calcd. for $C_{18}H_{18}O_4N_4$: C, 61.0; H, 5.12; N, 15.8. Found: C, 60.9; H, 5.14; N, 16.2.

Preparation of N,N'-Oxalylbis-(L-alanine anilide).—To 25 ml. of 0.5 *M* acetate buffer, *p*H 4.7, were added 0.3 g. of oxalylbis-(L-alanine), 0.5 g. of aniline, 0.08 g. of cysteine hydrochloride and 1.5 ml. of the papain extract. After incubation for 4 days at 40°, the precipitated anilide was removed by filtration, washed, and recrystallized from DMF–water, m.p. 305° dec., $[\alpha]^{25}D$ −11.5° (*c* 1.0, DMF).

It was somewhat more convenient to start with the ester instead of the free acid. Thus 0.90 g. (0.003 mole) of oxalylbis-(L-alanine ethyl ester) was shaken with 20 ml. of 0.7 *N* sodium hydroxide until solution was complete. The *p*H was then adjusted to 4.7 with glacial acetic acid and to the solution were added 0.96 g. (0.01 mole) of aniline, 0.15 g. of cysteine hydrochloride, and 4 ml. of the papain extract. After the addition of enough water to fill the vessel (a 40-ml. centrifuge tube) and stoppering tightly, the incubation was carried out at 35° for 4 days. In this case, the yield of anilide was 0.225 g. (19%), m.p. 305° dec.

The same product was obtained when the crude mixture of isomers of oxalylbis-(*dl*-alanine) was used as the substrate. A mixture of 1.5 g. (0.0065 mole) of the substrate, 2.4 g. (0.026 mole) of aniline, 0.15 g. of cysteine hydrochloride, and 3 ml. of the papain extract in 40 ml. of the acetate buffer after 2 days at 40°, yielded 0.225 g. (9%) of the anilide, m.p. 305° dec., $[\alpha]^{25}D$ −11.3° (*c* 1.0, DMF).

Anal. Calcd. for $C_{20}H_{22}O_4N_4$: C, 62.9; H, 5.80; N, 14.7. Found: C, 63.1; H, 5.69; N, 14.6.

Admixtures of this product and those obtained above from the L-isomer showed no depression in m.p.

When oxalylbis-(D-alanine) was subjected to the same reaction conditions, no precipitate was obtained.

Similarly, when the insoluble *meso*-oxalylbis-(alanine) was subjected to the same reaction conditions, no precipitate was obtained. However, after removal of this first fraction from the crude mixture of isomers, subsequent crops isolated by concentrating the mother liquor always produced some anilide under the above reaction conditions, demonstrating the presence of oxalylbis-(L-alanine), and therefore the racemate, in these more soluble fractions.

HOUSTON 25, TEXAS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, FORDHAM UNIVERSITY]

The Action of Fish Tissue on Thiamin. III.[1] The Further Elucidation of the Structure of Icthiamin[2-4]

BY EDWARD E. KUPSTAS AND DOUGLAS J. HENNESSY

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Chromatographic studies show that hypotaurine (2-aminoethanesulfinic acid) is formed in the reaction of icthiamin with hydroxide and with bisulfite in the presence of hydroquinone. Exhaustive drying indicates that icthiamin dihydrobromide is a monohydrate. These facts together with infrared absorption characteristic of the sulfone group and earlier information suggest that icthiamin is 4-amino-5-(2-aminoethanesulfonyl)-methyl-2-methylpyrimidine.

The presence in icthiamin of a (4-amino-2-methyl-5-pyrimidyl)-methyl moiety was reported in an earlier publication from this Laboratory.[1b] Evidence for the identity of an aliphatic fragment formed by a nucleophilic cleavage of icthiamin is now presented together with a structure for icthiamin.

In a preliminary report, Hennessy and Warner[5] had assigned the formula $C_8H_{16}N_4O_3S\cdot 2HCl$ to icthiamin dihydrochloride. Barnhurst and Hennessy later assigned the formula $C_8H_{14}N_4O_3S\cdot 2HX$ to the dihydrohalides despite the better agreement of the hydrogen analyses with the earlier formula. Support for the latter formula included the almost exact correspondence of the analytical data obtained on the dipicrate with that calculated for $C_8H_{14}N_4O_3S\cdot 2C_6H_3N_3O_7$ and the lack of any reasonable structure which could be assigned to the formula of Hennessy and Warner.

The isolation of taurine and 4-amino-2-methyl-5-pyrimidine-methanesulfonic acid from the bisulfite cleavage of icthiamin[6] at first suggested I or II, each having the formula $C_8H_{14}N_4O_3S$, as possible structures for icthiamin.

The failure of taurine to effect the destruction of thiamin in the presence of dialyzed clam tissue while many other nucleophilic reagents were quite effective seemed to militate against I.

When the dissociation constants of the conjugate acids of the aliphatic amino group of I and II were calculated using ammonia as the reference base according to a method described by Branch and Calvin,[7] pK_b values of 7.2 and 5.6, respectively, are obtained as compared to the value of 6.6 actually observed for icthiamin.[6] Better agreement, *i.e.*, 7.0, is found when the pK_b is calculated for the aliphatic amino group of a compound having structure III, whose hydrate agrees with the formula of Hennessy and Warner, $C_8H_{16}N_4O_3S$.

However, the failure of the icthiamin salts to lose water of hydration under mild conditions of dehydration and the synthesis[6] of what was believed to be 4-amino-5-(2-aminoethanesulfonyl)-methyl-2-methylpyrimidine (III) which was not identical with icthiamin appeared to eliminate this as a possible structure for icthiamin.

Icthiamin dihydrohalides when exhaustively

(1) Papers I and II, J. D. Barnhurst and D. J. Hennessy, (a) THIS JOURNAL, **74**, 353 (1952); (b) **74**, 356 (1952).

(2) This work was aided by a grant from the Williams-Waterman Fund.

(3) Presented in part before the Division of Biological Chemistry, American Chemical Society, 126th Meeting, New York, September, 1954.

(4) This paper is based on a portion of a thesis submitted by E. E. Kupstas to the Graduate School in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(5) D. J. Hennessy and S. Warner, Abstracts, 109th Meeting, American Chemical Society, Atlantic City, N. J., April 1946.

(6) J. D. Barnhurst, Thesis, Fordham University, 1951.

(7) G. E. K. Branch and M. Calvin, "The Theory of Organic Chemistry," Prentice-Hall, Inc., New York, N. Y., 1941, p. 203.