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The Use of Amino Acid Oxidases for the Small-scale Preparation of the Optical Isomers of Amino Acids

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Racemic amino acids at the 10-mmole level or less have been subjected to the action of *Crotalus adamanteus* venom L-amino acid oxidase and of hog renal D-amino acid oxidase, and the respective insusceptible optical isomers of the amino acids subsequently isolated and tested for chemical, optical and chromatographic purity. Thirteen D-isomers were so prepared through the action of the former enzyme, and nine L-isomers through the latter. All were found to be pure, the optical purity measured being in excess of 99.9%. In order for this level to be attained in the reacion of phenylalanine and of hydroxyproline with D-amino acid oxidase, it was necessary to have cyanide present. It was not possible, even in the presence of cyanide, to obtain sufficiently pure L-isomers of tyrosine and tryptophan.

Introduction

Enzymatic methods for the preparation of the optical antipodes of α-amino acids from the corresponding racemate fall into two general categories, (a) those based upon a preliminary synthesis of some derivative of the racemate, *i.e.*, N-acyl, ester or amide, which is subsequently asymmetrically hydrolyzed by the appropriate enzyme, and (b) those based upon a direct asymmetric oxidation or decarboxylation of one or the other antipode of the racemate.[1] The former constitutes a true resolution in the sense that both optical antipodes are separately recovered at the end of the enzymatic reaction, whereas in the latter approach one of the two antipodes is selectively destroyed. A general procedure based upon (a) which has been developed in this Laboratory[1,2] over the past several years has lent itself readily to the production of the optical isomers of the amino acids in quantity, for it has been possible by working on no more than a laboratory scale to prepare about 1 kilogram of L-isomer with an optical purity in excess of 99.9%[3] per day. The yield of the N-acyl or amide derivative employed in this procedure is usually 60–80%, and that of the L- or D-amino acid about the same, so that the over-all yield from the racemic amino acid may run from 30 to 60%.

On a scale of starting material of 1 g. or less, such as is provided frequently by the synthesis of amino acids containing isotopes, the problem of first preparing a satisfactory derivative and then isolating the products of the enzymatic reaction presents many difficulties in the way of economical recovery. It was therefore considered of value to turn to procedure (b), and to investigate the use of optically specific and available amino acid oxidases for the purpose of preparing the amino acid isomers on a small scale, with yields not greatly below, and with standards of purity no lower than, those obtained on a larger scale by procedure (a). If both antipodes of an amino acid are required, the use of the oxidases because of the selective destruction of one of the antipodes, limits the maximum yield to 50% of the racemate as compared with procedure (a). Procedure (b) has been employed with *Proteus vulgaris* L-amino acid oxidase to obtain D-methionine[4]; with renal D-amino acid oxidase to obtain L-alanine,[5,6] L-methionine[5] and L-proline[7]; and with bacterial decarboxylases to obtain D-glutamic acid[8] and D-lysine.[9]

The present, systematic studies have involved the use of *Crotalus adamanteus* (rattlesnake) venom L-amino acid oxidase to obtain the D-optical isomers of several amino acids, and of a hog renal D-amino acid oxidase preparation to obtain the L-isomers. Kinetic studies earlier reported for these enzymes and substrates were useful in preparing the preliminary conditions for the reaction.[10]

TABLE I

INITIAL QUANTITIES OF *Crotalus adamanteus* VENOM AND OF LYOPHILIZED HOG KIDNEY D-AMINO ACID OXIDASE EMPLOYED IN EACH REACTION VESSEL CONTAINING 10 MILLIMOLES OF RACEMIC AMINO ACID

Racemate	*Crotalus adamanteus*, g.	Lyophilized D-amino acid oxidase, g.
Alanine	0.5	0.833
Arginine·HCl	.8	..
Aspartic acid	.5	..
Glutamic acid	.5	..
Histidine·HCl	.23	..
L-Hydroxyproline plus D-allo-hydroxyproline (app. 1:1)	...	9.0
L-Isoleucine plus D-allo-isoleucine (app. 1:1)	0.11	2.8
Leucine	0.1	2.0
ε-Carbobenzoxylysine	1.0	..
Methionine	0.03	1.0
Phenylalanine	0.04	1.7
Proline	..	0.86
Serine	..	6.0
Tryptophan	0.04	(6.0)
Tyrosine	.17	(9.0)
Valine	.83	0.85

(1) J. P. Greenstein, *Adv. Prot. Chem.*, **9**, 121 (1954).

(2) S. M. Birnbaum, M. Winitz and J. P. Greenstein, *Archiv. Biochem. Biophys.*, **60**, 496 (1956); M. Winitz, L. Bloch-Frankenthal, N. Izumiya, S. M. Birnbaum, C. G. Baker and J. P. Greenstein, THIS JOURNAL, **78**, 2423 (1956); R. W. Wade, S. M. Birnbaum, M. Winitz, R. J. Koegel and J. P. Greenstein, *ibid.*, **79**, 648 (1957); R. Marshall, M. Winitz, S. M. Birnbaum and J. P. Greenstein, *ibid.*, **79**, 4438 (1957); L. Benoiton, M. Winitz, S. M. Birnbaum and J. P. Greenstein, *ibid.*, **79**, 6192 (1957).

(3) A. Meister, L. Levintow, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **192**, 535 (1951).

(4) P. K. Stumpf and D. E. Green, *ibid.*, **153**, 387 (1944).

(5) R. Duschinsky and J. Jeanneret, *Compt. rend.*, **208**, 1359 (1939).

(6) O. K. Behrens, *J. Biol. Chem.*, **141**, 465 (1941).

(7) M. R. Stetten and R. Schoenheimer, *ibid.*, **153**, 113 (1944).

(8) M. N. Camien, L. E. McClure and M. S. Dunn, *Arch. Biochem.*, **28**, 220 (1950).

(9) A. Neuberger and F. Sanger, *Biochem. J.*, **37**, 515 (1943); **38**, 125 (1944).

(10) J. P. Greenstein, S. M. Birnbaum and M. C. Otey, *J. Biol. Chem.*, **204**, 307 (1953).

TABLE II

CHARACTERISTICS OF THE AMINO ACID ISOMERS PREPARED

Amino acids	Yield, %[a]	Analyses, % Calculated C	Calculated H	Calculated N	Found C	Found H	Found N	$[\alpha]_D$[b]
D-Alanine	68	40.44	7.92	15.72	40.64	8.11	15.56	−14.6
D-Arginine·HCl	37	34.20	7.17	26.59	34.37	7.25	26.31	−21.8
D-Aspartic acid	58	36.09	5.30	10.52	35.97	5.52	10.64	−24.6
D-Glutamic acid	79	40.81	6.16	9.52	40.91	6.25	9.34	−30.3
D-Histidine·HCl monohydrate[c]	80	34.37	5.76	20.04	34.21	5.96	19.81	−10.29
D-Alloisoleucine	83	54.94	9.99	10.67	54.85	10.02	10.69	−37.8
D-Leucine	71	54.94	9.99	10.67	54.68	9.77	10.65	−15.1
ε-Carbobenzoxy-D-lysine	70	59.98	7.19	9.99	59.83	7.33	10.12	− 5.65[d]
D-Lysine·HCl	..	39.45	8.27	15.33	39.27	8.27	15.32	−20.6[f]
D-Methionine	79	40.25	7.43	9.39	40.08	7.33	9.27	−23.9
D-Phenylalanine[e]	64	65.51	6.81	8.48	65.43	6.71	8.56	+33.9[g]
D-Tryptophan	39	64.69	5.92	13.72	64.30	5.85	13.57	+31.7[h]
D-Tyrosine	77	59.66	6.12	7.73	59.55	6.15	7.72	+ 7.56
D-Valine	68	51.26	9.46	11.96	51.13	9.41	12.07	−26.4
L-Hydroxproline	56	45.79	6.91	10.68	45.74	6.92	10.74	−75.4[g]
L-Alanine	92	40.44	7.92	15.72	40.36	8.06	15.59	+14.1
L-Isoleucine	70	54.94	9.99	10.67	54.71	9.91	10.57	+37.5
L-Leucine	92	54.94	9.99	10.67	54.70	9.80	10.81	+15.6
L-Methionine	70	40.25	7.43	9.39	40.24	7.56	9.35	+23.1
L-Phenylalanine	74	65.43	6.71	8.48	65.41	6.90	8.58	−33.2
L-Proline	40	52.16	7.88	12.17	51.90	7.95	12.15	−83.8[g]
L-Serine	50	34.28	6.71	13.33	34.00	6.81	13.35	+14.6[i]
L-Valine	86	51.26	9.46	11.96	51.10	9.49	12.06	+26.7

[a] The yields reported are for analytically pure products. [b] Optical rotations were determined with 2% solutions in 5 *N* HCl, at temperatures between 24 and 28°, unless stated otherwise. The optical purity determined for each isomer[c] was in all cases >99.9%. [c] When dried at 140° and 1 mm. for 12 hours, histidine hydrochloride monohydrate loses water and turns brown. *Anal.* Calcd. for $C_6H_{10}N_3O_2Cl$: C, 37.60; H, 5.26; N, 21.92. Found: C, 37.90; H, 5.30; N, 21.64. [d] 2% solution in 1 *N* NaOH. [e] D- (or L)-phenylalanine when recrystallized from water–ethanol mixture and dried at 25° and 1 mm. analyzes for phenylalanine hemihydrate. *Anal.* Calcd. for $C_9H_{11}NO_2 \cdot {}^1/_2H_2O$: C, 62.05; H, 6.94; N, 8.04. Found: C, 62.35; H, 6.95; N, 8.12. When dried at 90° under the same conditions it loses the water of crystallization. [f] 4% solution in 5 *N* HCl. [g] 2% aqueous solution. [h] 0.5% aqueous solution. [i] 2% solution in 1 *N* HCl.

Isolation and purification of the product in each case was followed by tests for analytical and optical purity, the latter by further incubation with the same enzyme which had yielded it by selective destruction of its enantiomer,[8] for in no case could it be assumed that the oxidative reaction necessarily went to completion merely because no more oxygen was consumed. Inasmuch as this report is concerned with the criteria of purity of the compounds obtained in order to establish the validity of the methods, and since a sufficient supply of each compound was needed for replicate analytical determinations, for optical rotation measurements and for measurements of optical purity, much greater quantities of the starting racemic amino acids were employed than the method itself required. At the conclusion of these studies, however, model experiments involving the use of only 1 mmole of DL-methionine were performed, the corresponding L- and D-isomers being obtained in yields of 93 and 85%, respectively.

Results and Discussion

The products of the oxidative reaction in all cases, except for proline and hydroxyproline, include ammonia, the corresponding α-keto acid and hydrogen peroxide. The action of the peroxide on the keto acid, unless the former is destroyed by catalase, leads to decarboxylation with the formation of carbon dioxide and a fatty acid containing one less carbon atom. *Crotalus adamanteus* venom contains no catalase and, if the α-keto acid is to be preserved intact, it is necessary to add catalase.[11] Kidney D-amino acid oxidase preparations in the relatively crude form used in the present experiments contain much catalase activity, and in order to inhibit the latter enzyme without affecting the former, cyanide may be employed.

Essentially no problems were encountered in the use of the snake venom L-amino acid oxidase for the preparation of the D-isomers from the respective racemates. Table I gives the initial amounts of venom employed per 10 mmoles of racemic substrate. In all cases, except for aspartic and glutamic acids, these amounts were sufficient to effect completion of the oxidation reaction in 18–24 hours. With the dicarboxylic amino acids twice the initial amount of venom and twice the incubation period were necessary to complete the oxidation, but in all cases the D-isomer finally isolated was optically pure (>99.9%), possessed the correct elemental analytical values and revealed $[\alpha]_D$ values in good agreement with those in the literature (Table II). These results suggested that the oxidation products, namely, α-keto acids in cases where catalase had been added or corresponding fatty acids with one less carbon atom where the catalase had been omitted, exerted no appreciable inhibition of the oxidative reactions. Only in the case of histidine was the presence of catalase found to be deleterious. Reference to Table II shows that D-alloisoleucine was prepared, but this occurred because

(11) A. Meister, *J. Biol. Chem.*, **190**, 269 (1951).

the starting material was the epimeric mixture of L-isoleucine plus D-alloisoleucine currently available on the market; DL-isoleucine was not available, but there is no reason to believe that any difficulties would be encountered in the isolation of D-isoleucine from this racemate after the action of the snake venom and oxygen. Among the protein amino acids whose D-isomers are missing in Table II are serine, threonine, hydroxyproline and cystine; the L-antipodes of the former three amino acids are completely or nearly completely resistant to the action of the snake venom, whilst the action of the venom on L-cystine, although quite rapid at first,[10] never appears to reach completion.

A quite different and more difficult situation was encountered in the use of hog renal D-amino acid oxidase for the preparation of the L-isomers from the racemates. The use of pyrophosphate buffer was found to be essential for the oxidative reaction to go to completion, whilst other buffers at the same *p*H such as Tris or veronal acetate were not effective for this purpose. The oxidation of the racemates of alanine, isoleucine, leucine, methionine, proline, serine and valine went to completion in 18–24 hours with the initial amounts of lyophilized D-amino acid oxidase described in Table I. In each instance, however, the reaction mixture was highly colored. Adsorption of the L-isomers on a Dowex-50 resin column followed by displacement with ammonia led to products still containing some color, and which were not analytically pure. Repeated crystallizations in the presence of Norit were ineffective in purifying the compounds. This difficulty was overcome by the subsequent use of a Dowex-1 anionic resin column which held back the colored and other associated impurities, save in the cases of L-proline and L-serine. The other L-isomers obtained through the successive use of these two resins were highly pure (Table II). L-Proline and L-serine isolated in a similar manner were still colored to some extent, but were finally obtained in the pure, colorless state by careful fractional recrystallization. Attempts to prepare the L-isomers of phenylalanine, tryptophan, tyrosine and hydroxyproline led to products largely contaminated with the respective D-isomers. Although the original reaction mixtures involving these amino acids no longer took up oxygen, and were inert to fresh enzyme, the compounds *isolated* from these mixtures and established in newly prepared mixtures, were quite reactive to freshly added enzyme and oxygen, ultimately slowing down, in their turn, short of the completed goal. The respective α-keto acids or other metabolic products, corresponding to these amino acids, formed from the susceptible D-isomers during the reaction, must apparently have brought the reactions to a halt. Renal D-amino acid oxidase as shown by Hellerman and his associates[12] is considerably inhibited by many aromatic compounds such as benzoic acid, phenylpyruvic acid, indole-2-carboxylic acid, etc. It would therefore be expected that the oxidation of the aromatic amino acids would be effectively hindered by the products of the reaction in each case.

In the case of phenylalanine, the corresponding α-keto acid is phenylpyruvic acid. Inasmuch as phenylpyruvic acid is a more powerful inhibitor of D-amino acid oxidase than is phenylacetic acid,[12,13] it was decided to conduct the oxidation of racemic phenylalanine in the presence of cyanide. The product from such an experiment contained 5% of the D-antipode, compared with over 20% when the cyanide was omitted. Reoxidation of this sample of L-phenylalanine with fresh D-amino acid oxidase, again in the presence of cyanide, gave optically-pure L-phenylalanine in good yield. Similar attempts to prepare L-tyrosine and L-tryptophan from the respective racemates in the presence or absence of cyanide, and after repeated oxidation of the isolated products in the presence of fresh enzyme, gave L-isomers of apparently increasing optical purity but with diminishing yields. It was considered impracticable, however, to extend the purification of these products any further by the present means.

DL-Hydroxyproline was not available, and therefore an epimeric mixture was prepared by the racemization of L-hydroxyproline in hot baryta solution according to the procedure of Leuchs and Geiger.[14] The resulting mixture of L-hydroxyproline and D-allohydroxyproline (app. 1:1) was then subjected to enzymatic oxidation. D-Allohydroxyproline and D-hydroxyproline are nearly equally susceptible to the action of D-amino acid oxidase.[10] The oxidation of the susceptible D-isomer of the epimeric mixture turned out to be incomplete despite the use of a large initial amount of the D-amino acid oxidase preparation. Recent studies by Radhakrishnan and Meister[15] have shown that the oxidation of D-hydroxyproline and of D-allohydroxyproline by purified D-amino acid oxidase (in the presence of catalase) leads to the formation of 2-pyrrolecarboxylic acid. That the reaction occurs *in vivo*, as well, was shortly thereafter shown by Letellier and Bouthillier.[16] As further shown by Radhakrishnan and Meister, the enzymatic oxidation of D-hydroxyproline *in the absence of catalase*, leads to the formation of β-hydroxy-γ-aminobutyric acid (under the same conditions D-proline yields γ-aminobutyric acid).[15] Preliminary studies of the enzymatic oxidation of D-hydroxyproline revealed a much greater consumption of oxygen in the presence than in the absence of added cyanide, and it appeared probable (a) that 2-pyrrolecarboxylic acid might be a powerful inhibitor of D-amino acid oxidase, whereas (b) β-hydroxy-γ-aminobutyric acid might affect the activity level of this enzyme little if at all. The preparation of optically and analytically pure L-hydroxyproline from the epimeric mixture was therefore conducted with a considerable amount of the D-amino acid oxidase preparation in the presence of an effective concentration of cyanide (Tables I and II).

(12) L. Hellerman, A. Lindsay and M. R. Bovarnick, *J. Biol. Chem.*, **163**, 553 (1946); W. R. Frisell, H. J. Lowe and L. Hellerman, *ibid.*, **223**, 75 (1956).

(13) J. R. Klein and H. Kamin, *ibid.*, **138**, 507 (1941).

(14) H. Leuchs and W. Geiger, *Ber.*, **41**, 1731 (1908).

(15) A. N. Radhakrishnan and A. Meister, *Federation Proc.*, **15**, 333 (1956); *J. Biol. Chem.*, **226**, 559 (1957).

(16) G. Letellier and L. P. Bouthillier, *Can. J. Biochem. Physiol.*, **34**, 1123 (1956).

Procedures for the testing of the optical purity of the L-isomers of phenylalanine, tyrosine, tryptophan[3] and hydroxyproline[10] with large amounts of D-amino acid oxidase have not hitherto been conducted in the presence of cyanide. The method, however, has been applied successfully in these cases because there has been no consumption of oxygen by the 1000 μmoles employed of each L-isomer, whilst there was appreciable if not quite quantitative consumption of oxygen in the simultaneous test with 1000 μmoles of the L-isomer plus 1 μmole of added, pure D-isomer. From the present results it is advisable, if the latter reaction is to be made more nearly quantitative, that it be conducted in the presence of added 0.005–0.01 M cyanide.

The inhibition of the D-amino acid oxidase preparation by 2-pyrrolecarboxylic acid and by phenylpyruvic acid was studied with DL-alanine as substrate, the oxidation product of which, pyruvic acid, has little or no effect on the activity of the enzyme. The results are given in Fig. 1. A consideration of the results by the usual procedure[17] indicates that the two inhibitors act by competition with the substrate. The Michaelis–Menten constants[18] were calculated from the data. The K_s value for DL-alanine of 3.5×10^{-3} mole per liter is of the same order of magnitude as that obtained by other workers.[12] The Ki values of 9.9×10^{-6} mole per liter and of 6.3×10^{-4} mole per liter for 2-pyrrolecarboxylic acid and for phenylpyruvic acid, respectively, suggest that the former is bound to the enzyme about 1,000 times more strongly, whilst the latter is bound about 10 times more strongly to the enzyme than is the substrate.

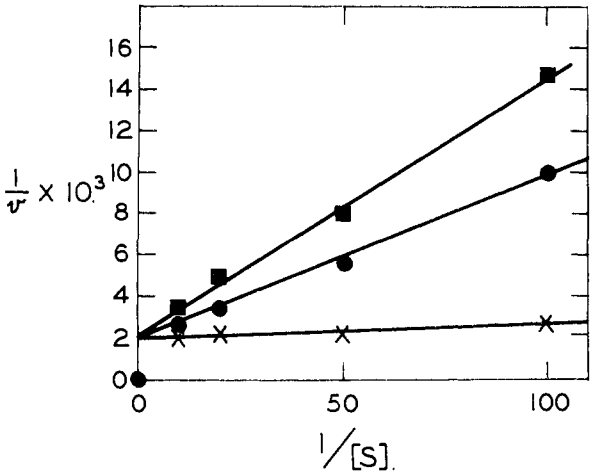

Fig. 1.—Effect of 10^{-4} M 2-pyrrolecarboxylic acid, ⊙, and of 10^{-2} M phenylpyruvic acid, ⊡, on the oxidation of DL-alanine by hog renal D-amino acid oxidase, T = 37°. Reaction without inhibitor represented by X. Reaction mixture consisted of 2 ml. containing DL-alanine substrate in 0.1 M pyrophosphate buffer at pH 8.1 and 0.1 ml. of catalase; the side-arm contained 0.4 g. of hog renal D-amino acid oxidase in 0.4 ml. of the buffer. KOH was present in the center cup. v = μl. of O_2 consumed per hour, S = molar concentration of susceptible D-isomer of the substrate at 2×10^{-1}, 4×10^{-2}, and 2×10^{-2} M.

The action of the D-amino acid oxidase preparation on the D-component of DL-arginine·HCl, DL-aspartic acid, DL-glutamic acid, DL-histidine·HCl, and ϵ-carbobenzoxy-DL-lysine was too weak in each case to permit a practicable preparation of the L-isomers of these amino acids. In any event, the arginase activity still inherent in the crude preparation would effectively convert the L-arginine component of the first-mentioned racemate into L-ornithine, whilst it is dubious whether the L-histidine component of DL-histidine would long maintain its integrity in the presence of the crude kidney preparation.

Experimental

Preparation of the D-Amino Acids.—The L-amino acid oxidase preparation was dried *Crotalus adamanteus* venom obtained from the Ross Allen Reptile Institute of Silver Springs, Florida. The venom was dissolved in the appropriate volume of 0.2 M *tris* buffer at pH 7.2, dialyzed against running tap water for 5–6 hours at 5°, and the solution centrifuged before use. Crystalline liver catalase was obtained from the Worthington Co. and its aqueous solution thoroughly dialyzed at 5°.

D-Alanine.—In the main compartment of a 125-ml. Warburg respirometer was placed a solution of 0.89 g. (0.01 mole) of DL-alanine in 30 ml. of water[19] together with 10 ml. of 0.2 M *tris* buffer at pH 7.2. Immediately thereafter was added 10 ml. of a dialyzed solution of the venom and 0.4 ml. of a solution containing 100 units of catalase.[20] To the center cup was added 2 ml. of 20% KOH provided with a filter paper strip. Inasmuch as it was necessary to prepare enough material for analytical, optical rotation and purity determinations in replicate, three such flasks and their contents were so prepared. The three flasks together with a control flask wherein the substrate was omitted were attached to the manometric assembly and shaken at 37° in an atmosphere of oxygen for 18 to 24 hours. At intervals the oxygen supply was shut off, the side-arms were closed, and the oxygen consumption noted. When no further oxygen was being consumed, 0.3 ml. of a freshly-prepared solution of the venom was added to the side-arm of each flask. After a brief equilibration period, the enzyme solution was tipped into the flask and the oxygen consumption noted. In this manner the end-point of the reaction was ascertained. At the end of the reaction the three substrate suspensions were combined, brought to isoelectric pH by addition of a few drops of glacial acetic acid, and concentrated *in vacuo* at 45–50° to about 30 ml. The concentrate was dialyzed with agitation against 4 changes of 150 ml. each of distilled water at intervals of 2 hours.[21] The combined dialysates were warmed with Norit and filtered, the filtrate and washings concentrated to a low bulk, and ethanol added to 80%. The D-alanine which appeared on chilling was recrystallized in the same manner. In the earlier studies of this reaction, the products in each flask were worked up separately in order to check the yields under these conditions, and to note the applicability of the method to small-scale preparations. At the 10-mmole level the isolation of the D-isomers was quite successful, the combined yield being little below that obtained from the combined reaction mixtures as described above. Optical purity[3] and rotation measurements were carried out on the analyzed product. These determinations required approximately 700 mg. (about 180 mg. for optical purity, 400 mg. for rotation, and 100 mg. for elemental analyses) or close to the 900 mg. yield of twice-crystallized and pure D-alanine finally obtained (*cf.* Table II).

D-Alloisoleucine, D-methionine, D-phenylalanine, D-tryptophan and D-valine were prepared from the corresponding racemates (the isoleucine racemate was a nearly equal mixture of D-isoleucine and D-alloisoleucine) in a manner similar to that described for D-alanine, with modifications

(17) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

(18) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913).

(19) All of the racemic amino acids employed herein were recrystallized before use.

(20) Catalase was omitted in the preparation of the D-isomers of aspartic acid, glutamic acid, histidine, alloisoleucine, lysine and methionine.

(21) For more insoluble amino acids the quantity of water employed for dialysis was considerably increased.

in regard to the amount of venom employed as noted in Table I. More water was also employed in the dialytic step for these compounds. Norit was not employed in the purification procedures for the aromatic amino acids.

D-Arginine.—This preparation was achieved in the same manner as with D-alanine, except that the final product was isolated as the benzylidine derivative,[22] and from this converted to the pure monohydrochloride.

D-Aspartic Acid and D-Glutamic Acid.—The corresponding racemates were brought into solution at neutrality by addition of LiOH, and the oxidation carried on as described for alanine. At the end of the usual 24-hour period the consumption of oxygen had apparently ceased. Addition of fresh venom revealed renewed oxygen uptake, so that the reaction was essentially uncompleted. Rather than continuing with the same digests and fresh enzyme, it was decided to isolate the crude products and resume the oxidation with fresh solutions. The concentrates obtained from the combined dialysates were adjusted by addition of 1 *N* HCl to *p*H 2.8, and the D-isomers isolated in the usual manner from water:ethanol in 1:4 ratio. Optical purity determinations revealed the presence of appreciable amounts of the respective L-isomers. The products were therefore each divided into 3 parts and subjected to reoxidation using the same amount of venom as before. The new products obtained thereby were optically pure.

D-Histidine.—The solution of racemic histidine·HCl was brought to neutrality by addition of 1 *N* LiOH prior to oxidation. If catalase is used in this reaction, the mixture assumes a strong cherry-red color which turns to a dark brown at the end, and the yields are remarkably poor. This phenomenon appears to be unique for histidine. In the absence of catalase the reaction proceeded normally and the D-histidine was isolated in good yield as the monohydrochloride monohydrate.

D-Lysine.—The substrate for this reaction was DL-ε-carbobenzoxylysine. Despite its insolubility the oxidation reaction proceeded normally to completion. At the end of the oxidation period the reaction mixtures were combined, brought to *p*H 5 with acetic acid, filtered with suction, and washed with ice-water. The precipitate, D-ε-carbobenzoxylysine, was recrystallized twice from water with the aid of Norit and Cello-cel to remove the coagulated venom protein. The product was catalytically hydrogenated and the D-lysine isolated first as the dihydrochloride and subsequently as the monohydrochloride.

D-Tyrosine.—Because of the very considerable insolubility of this amino acid and its lack of wetting, the shaking procedure in the Warburg vessel was initially ineffective. The racemic tyrosine (1.81 g. = 10 mmoles) was therefore suspended in a 500-ml. volumetric cylinder in a mixture of 120 ml. of water, 40 ml. of 0.2 *M tris* buffer at *p*H 7.2, 0.4 ml. of dialyzed solution containing 100 units of catalase and 40 ml. of a dialyzed solution of venom. Three cylinders with the above contents and one serving as blank without substrate were placed in a bath at 37°. A steady stream of oxygen was bubbled through each reaction mixture by means of gas dispersion tubes, and foaming was controlled by occasional addition of caprylic alcohol. Care was taken to prevent climbing of tyrosine crystals up to the walls of the cylinders. After 24 hours of oxidation, 2-ml. representative samples were withdrawn from each mixture and transferred to Warburg respirometers. The end-point of the reaction was checked with fresh venom solution, and it was found that there was still some unoxidized L-tyrosine present. Fresh venom solution to the same amount was added and the reaction continued for another 24-hour period when it was complete. The mixtures were combined, brought to *p*H 5, the D-tyrosine filtered and washed. The filtrate was concentrated to about 100 ml. *in vacuo* and chilled for several hours. The first and second crops of D-tyrosine were combined and recrystallized from water with the aid of Norit to remove venom protein.

D-Leucine.—The oxidation reaction with DL-leucine was carried out in a manner similar to that described for DL-tyrosine, except that the leucine was suspended in each cylinder in a mixture of 60 ml. of water, 20 ml. of *tris* buffer, 20 ml. of venom solution and 0.4 ml. of catalase solution (100 units). The reaction was completed readily in less than 24 hours, and the mixtures were combined, adjusted to *p*H 5, and condensed *in vacuo* to about 35 ml. The concentrate was dialyzed against 4 changes of 300 ml. of distilled water each time at 2-hour intervals. The combined dialysates were concentrated *in vacuo* to about 200 ml. and the concentrate poured onto a Dowex-50 resin column, 4 × 30 cm. (200–400 mesh) in the hydrogen form. After thorough washing of the column with water to neutrality, the D-leucine was displaced by a 1 *N* ammonium hydroxide solution and collected. The eluate was evaporated to dryness *in vacuo*, the residue taken up in water, and the evaporation repeated. This procedure was repeated again in order to remove the last trace of ammonia. The residue was dissolved in about 100 ml. of water and the solution poured onto a Dowex-1 resin column, 2.5 × 22 cm. (50–100 mesh) in the hydroxyl form. After washing with water to neutrality, the D-leucine was eluted with 1 *N* acetic acid. The collected ninhydrin-positive fractions were combined, filtered, and evaporated *in vacuo* several times to dryness, each time after dissolution of the residue in water. The residue was taken up in about 50 ml. of hot ethanol and the solution allowed to chill for several hours. The colorless crystals of D-leucine were collected by filtration, washed with chilled ethanol, and recrystallized from aqueous ethanol.

Preparation of the L-Amino Acids.—The D-amino acid oxidase preparation was derived from hog kidney. Some 600 g. of previously frozen kidneys was minced and extracted with about 4 l. of cold C.P. acetone in a Waring Blendor. The suspension was filtered and the filter-cake extracted as above. The final preparation was dried *in vacuo* over P_2O_5. Several batches were prepared in this manner, and yielded an average of 130 g. of acetone powder from 600 g. of raw kidney. An amount of 65 g. of this powder was extracted with 350 ml. of cold distilled water in a Waring Blendor, the extract was centrifuged in a Spinco centrifuge at 18,000 r.p.m. for 30 minutes, and supernatant set aside in the cold. The solid residue was re-extracted as above, and the combined supernatant solutions lyophilized and stored at −5°. The average yield of lyophilized material was 18 g.

Because quite large amounts of this crude enzymic preparation were employed in the oxidation of the various amino acids, with the consequent danger of contamination of the isomers by amino acids liberated by autolysis of the enzyme, each of the L-isomers in the final, purified state (Table II) was subjected to two-dimensional paper chromatography using 40-μg. samples. One solvent was phenol–NH_3 or phenol saturated with 10% trisodium citrate, the other was formic acid:H_2O:*sec*-butyl alcohol (15:15:70). In every case of the L-amino acids described in Table II, only a single ninhydrin-reactive spot could be developed except in the case of L-methionine which showed faint evidence for the usual presence of the corresponding sulfoxide.

L-Alanine.—In a 500-ml. volumetric cylinder were placed 0.89 g. (10 mmoles) of DL-alanine, 40 ml. of water and 10 ml. of a solution containing D-amino acid oxidase (*cf.* Table I) in 0.1 *M* sodium pyrophosphate buffer at *p*H 8.2. Three such cylinders with similar contents plus one cylinder in which the substrate was omitted were placed in a constant temperature water-bath at 37°, and a steady stream of oxygen bubbled through the reaction mixtures, caprylic alcohol being added to reduce foaming. The *p*H was maintained at 8.0 by appropriate addition of LiOH solution. The end-point of the reaction was determined as in the case of D-tyrosine described above. When this was established (less than 24 hours incubation) the combined mixtures were brought to *p*H 5, evaporated *in vacuo* to about 30 ml., and dialyzed against 4 changes of 500 ml. of water each at 2-hour intervals. The combined dialysates were evaporated *in vacuo* to a small bulk and the solution passed through first a Dowex-50 resin column and then a Dowex-1 resin column in acetate form as described in the case of D-leucine above. Two crystallizations from water–ethanol (1:4) yielded a pure product of L-alanine.[23]

L-Isoleucine, L-leucine, L-methionine, L-proline, L-serine and L-valine were obtained from the respective racemates

(22) M. Bergmann and L. Zervas, *Z. physiol. Chem.*, **152**, 282 (1926); **172**, 277 (1927); S. M. Birnbaum, M. Winitz and J. P. Greenstein, *Arch. Biochem. Biophys.*, **60**, 496 (1956).

(23) The eluate from the acid Dowex-50 column contains yellowish impurities which seemingly cannot be removed readily from the amino acid, and which are essentially degradation products of the D-amino acid oxidase preparation. These contaminants are removed on the Dowex-1 resin column.

by treatment similar to that described for L-alanine, except that in the cases of isoleucine, leucine, methionine and valine, twice the volume of water and of buffer solution was employed. Each of the isomers except proline and serine was readily crystallized from water–ethanol in white, pure condition. L-Proline and L-serine come off the Dowex-1 resin (in hydroxyl or acetate form) in a highly colored state. Further treatment with the Dowex-1 resin removed some but not all of the colored impurity. Proline was dissolved in hot absolute ethanol, whilst serine was dissolved in hot water and treated with hot ethanol to 85%; on cooling to room temperature each solution deposited a small amount of a highly colored oil, leaving the supernatant solution clear and colorless. The supernatant solutions were each decanted from the oil and chilled at 0° for 1–2 days, whereby pure, colorless crystals of L-proline and of L-serine separated. Repetition of the preparation of these two isomers in the presence of 0.01 *M* KCN led to the isolation of L-serine and of L-proline in yield and state of purity reported in Table II.

L-Phenylalanine.—The racemic phenylalanine was subjected to oxidation in the same manner as that described for L-isoleucine but with the addition of KCN to 0.01 *M* final concentration to each reaction mixture. The product obtained after a 24-hour period of incubation when the oxygen consumption was apparently at an end was examined for optical purity[3] and revealed the presence of approximately 5% of the D-isomer. (If cyanide is omitted from the reaction mixture, the product at this stage contains about 20% of the D-form.) It was subjected to reoxidation in twice the volume of water and buffer as before, with twice the amount of enzyme, and once more in the presence of 0.01 *M* cyanide. After a further 24-hour period of oxidation at 37°, the isolated product after crystallization from water–ethanol was analytically and optically pure (Table II). In the case of DL-tyrosine oxidized by D-amino acid oxidase in the presence or absence of cyanide, the product in either case contained about 30% of the D-isomer; reoxidation in the presence of 0.01 *M* cyanide yielded a product still grossly contaminated with the D-form. A quite similar situation appeared to hold in the case of DL-tryptophan. It appeared probable that in these cases the lower fatty acid was about as inhibitory as the α-keto acid, and attempts to prepare the L-isomers of tyrosine and of tryptophan by the present procedures were therefore abandoned.

L-Hydroxyproline.—DL-Hydroxyproline is not readily available, and to test the present procedure for the preparation of the L-isomer, a suitable substrate was obtained by the epimerization of L-hydroxyproline in hot baryta solution. Leuchs and Geiger[14] prepared the epimeric mixture of L-hydroxyproline and D-allohydroxyproline (app. 1:1) in this manner, isolating the mixture after quantitative removal of barium ion with sulfate. Their procedure was modified to the extent of heating a solution of 20 g. of L-hydroxyproline in 250 ml. of water containing 150 g. of crystalline baryta at 120° for 24 hours, diluting with water and removing excess barium ion with CO_2 gas, and pouring the filtrate on a Dowex-50 resin (H^+) column, followed by a water wash. This was followed by 1 *N* NH_4OH until the ninhydrin reaction was negative, the eluate was evaporated to dryness to remove ammonia, and the crystalline, ammonia-free residue dried; yield 95%, $[\alpha]^{25}D$ −6.4° (*c* 2, H_2O).[24]

The epimeric mixture was subjected to oxidation for 24 hours as described above for L-alanine, with the amount of enzyme given in Table I, and in the presence of 0.005 *M* potassium cyanide. The L-isomer was isolated in the usual way and, after crystallization from water in the presence of excess ethanol, was found to be analytically and optically pure (Table II).

Preparation of L- and D-Methionine from 1 Mmole of DL-Methionine.—The oxidation of 149 mg. of DL-methionine was conducted in a 125-ml. Warburg vessel in the presence of one-tenth the volume of solvents, buffers, and amount of respective enzymes described above for the larger preparations. At the end of the oxidative reaction, the dialysis step was undertaken as above and the combined dialysates from either the L- or D-amino acid oxidase reaction treated successively on Dowex-50 and Dowex-1 resin columns as described above for D-leucine. The yield of D-methionine was 69.6 mg. (93%) and of L-methionine 63.0 mg. (85%).

(24) L-Allohydroxyproline treated in the same way yielded an epimeric mixture (app. 1:1) of L-allohydroxyproline and D-hydroxyproline with $[\alpha]^{25}D$ +7.4° (*c* 2, H_2O).

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[CONTRIBUTION FROM THE STERLING-WINTHROP RESEARCH INSTITUTE]

The Cleavage of 3-Tropanyl Chloride with Potassium Cyanide[1]

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The tropanyl chloride derived from tropine and thionyl chloride reacted with potassium cyanide in aqueous alcoholic solution to give a mixture of 2-allyl-5-cyano-1-methylpyrrolidines. The same substances are obtained when the toluenesulfonate of pseudo-tropine is used instead of the chlorotropane. This mixture of nitriles was converted by phenylmagnesium bromide to a pair of 2-allyl-1-methyl-5-phenylpyrrolidines. Quaternization, followed by a Hofmann degradation afforded a styrene which was reduced to the corresponding dihydro base, 4-dimethylamino-1-phenylheptane. The latter was synthesized by an independent route. This cleavage appears to be a variant of the 1–3 cleavages recently discussed by Grob[5] and therefore resembles the quinine–niquine transformation.[8]

In the course of some preparative work the need arose for a tropanyl nitrile. At that time an adequate supply of tropine was available and we transformed this alcohol to the corresponding chloride[2] in chloroform solution. The insoluble hydrochloride was converted to the free base before it was subjected to the action of aqueous alcoholic potassium cyanide. A sharply boiling liquid was obtained, whose analysis agreed with the formula $C_9H_{14}N_2$, but the infrared spectrum (Fig. 1) showed two cyanide bands in the 4.50 μ region and a band of moderate intensity at 6.08 μ. Further, the fingerprint region consisted of a series of blunted rather than crisp peaks which was also suggestive that a mixture rather than a pure compound was at hand. The nitrile group was converted readily to the corresponding methyl ester, but as in the case of the parent nitrile(s) it was difficult to obtain pure derivatives.

The action of phenylmagnesium bromide on the nitrile mixture resulted in the formation of another liquid, the analysis of which suggested the formula $C_{14}H_{19}N$. The over-all reaction appeared to be the replacement of the cyano group by a phenyl radical. This behavior is typical of α-dialkylaminoacetonitriles,[3] and it turned out that this structural

(1) A preliminary communication has been published; S. Archer, T. R. Lewis and Bernard Zenitz, THIS JOURNAL, **79**, 3603 (1957).

(2) M. Polonovski and M. Polonovski, *Bull. soc. chim.*, [4] **45**, 305 (1929).

(3) P. Bruylants, *Bull. soc. chim. Belg.*, **33**, 467 (1924); *C. A.*, **19**, 288 (1925).