Thus the acid-dissociation of this type of compound may be explained on the basis of either "protonated" or "half-bond" intermediates.

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THE CALCIUM CHLORIDE AND HYDROXYL ION CATALYZED HYDROLYSIS OF SEVERAL ACYLATED α-AMINO ACID ESTERS

Sir:

The availability of a pH-Stat[1,2] has permitted us to determine the base catalyzed hydrolysis of several acylated α-amino acid esters, to the acylated α-amino acid and alcohol, in the presence of calcium chloride and in the absence of conventional buffers. Under these conditions it has been found that the initial rates of hydrolysis are first order with respect to ester and hydroxyl ion but decrease from first order with respect to calcium chloride as the mean ion activity of the latter species is increased. From the reactions $S + M \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MS$; $MS + OH^- \xrightarrow{k_3} M + P$; $S + OH^- \xrightarrow{k_4} P$, it follows that $v' = d[P]/dt = k_3[MS][OH^-] + k_4[S][OH^-]$. Experiments conducted under conditions where [M] >> [MS], when [M] is the mean ion activity of calcium chloride,[3] allowed us to establish the relationship $v' = k_3[MS][OH^-] + k_4[\sigma][OH^-] - k_4[MS][OH^-]$, when $[\sigma] = [S] + [MS]$. Assuming a steady state process for MS, it follows that $v' - k_4[\sigma]\cdot[OH^-] = v = \{(k_3 - k_4)[OH^-][M][\sigma]\}/\{[M] + ((k_2 + k_3[OH^-])/k_1)\}$. Since $k_4[\sigma][OH^-]$ was determinable and the reactions were first order with respect to hydroxyl ion, $k_2 >> k_3[OH^-]$ and the second term in the denominator of the preceeding equation may be taken as the equilibrium constant $K = k_2/k_1 = [M][S]/[MS]$. Setting $k_3' = k_3[OH^-]$, $k'_4 = k_4[OH^-]$ and $k_3'' = (k_3' - k_4') = (k_3 - k_4)[OH^-]$ it follows that $[M]/(v/[\sigma]) = ([M]/k_3'') + K/k_3''$ and a plot of the left hand term *vs.* [M] will give a line of slope $1/k_3''$ and intercept K/k_3''. Such linear relationships were observed and led to the values of K and k_3 given in Table I. The constancy of K, for benzoylglycine methyl ester at *p*H 7.90 and 8.40, confirms the conclusion that $k_2 >> k_3[OH^-]$ and $K = k_2/k_1$.

While calcium chloride acting in conjunction with hydroxyl ion is not a very efficient catalyst for the hydrolysis of acylated α-amino acid esters, it is just this property that permits the separate evaluation of K and k_3. In addition the use of a *p*H-Stat[1,2] has avoided complications arising from specific buffer effects such as those encountered by Bender and Turnquest[4] in their studies on the cupric ion catalyzed hydrolysis of several α-amino acid esters, a reaction examined earlier by Kroll.[5]

(1) C. F. Jacobsen and J. Leonis, *Compt. rend. trav. lab. Carlsberg, Ser. Chim.*, **27**, 333 (1951).

(2) J. B. Nielands and M. D. Cannon, *Anal. Chem.*, **27**, 29 (1955).

(3) R. H. Stokes, *Trans. Faraday Soc.*, **44**, 295 (1948).

(4) M. L. Bender and B. W. Turnquest, THIS JOURNAL, **79**, 1889 (1957).

(5) H. Kroll, *ibid.*, **74**, 2036 (1952).

TABLE I
VALUES OF K AND k_3[a,b]

Ester	*p*H	K, M	k_3, M^{-1}, sec.$^{-1}$
Benzoylglycine methyl ester	7.90	25 ± 2	680 ± 30
	8.40	25 ± 2	670 ± 30
Acetyl-L-valine methyl ester	7.90	2.8 ± 0.1	7.2 ± 0.2

[a] In aqueous solutions at 25.0° and containing calcium chloride. [b] Constants evaluated from a minimum of eight experiments conducted under conditions where the concentration of ester was varied from 5 to 40 × 10^{-3} M and that of calcium chloride from 0.6 to 3.0 M.

The observation that a more stable MS complex may react with hydroxyl ion to give hydrolysis products at a slower rate than a less stable MS complex, *cf.* Table I, is but one example of the information that can be gained from knowledge of the constants K and k_3. The fact that the reaction system described in this communication is an almost complete analog of the simple enzyme-catalyzed reaction creates further interest in a system which in its own right is of importance in developing an understanding of the characteristics of an important class of solvolytic reactions.

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SYNTHESIS OF AMINO ACIDS CATALYZED BY AMINO ACID OXIDASES[1]

Sir:

The oxidation of amino acids by the general L- and D-amino acid oxidases[2] has been considered to be an irreversible process involving an unstable imino acid intermediate which hydrolyzes spontaneously to the corresponding α-keto acid and ammonia in accordance with the reactions

$$RCHNH_2COOH + \text{Enzyme–FAD} \longrightarrow RC{=}NHCOOH + \text{Enzyme–FADH}_2$$

$$RC{=}NHCOOH + H_2O \longrightarrow RCOCOOH + NH_3$$

$$\text{Enzyme–FADH}_2 + O_2 \longrightarrow \text{Enzyme–FAD} + H_2O_2$$

In the presence of oxygen the reaction will proceed in the direction of α-keto acid formation because of continuous oxidation of enzyme-bound reduced coenzyme.

We have carried out experiments in which a purified amino acid oxidase preparation and an amino acid were incubated with the α-keto acid analog of another amino acid substrate. Under anaerobic conditions, L-amino acid oxidase and D-amino acid oxidase catalyzed the formation of L- and D-amino acid isomers, respectively, from the corresponding α-keto acids. Thus, D-alanine was formed when D-amino acid oxidase,[3] pyruvate, and D-phenylalanine were incubated in the absence of oxygen; the reverse reaction was observed with D-alanine

(1) Supported in part by grants from the National Science Foundation and from the National Heart Institute, National Institutes of Health, Public Health Service.

(2) H. A. Krebs, in J. B. Sumner and K. Myrbäck, "The Enzymes," Vol. II, Part 1, Academic Press, Inc., New York, N. Y., 1951, p. 499.

(3) Prepared from sheep kidney by the procedure of E. Negelein and H. Brömel, *Biochem. Z.*, **300**, 225 (1939); the purified preparation was treated with an excess of FAD, and then dialyzed against 0.1 M sodium pyrophosphate buffer (*p*H 8.3) to remove free FAD and free ammonia.

and phenylpyruvate. The synthesis of D-valine, D-methionine, D-pipecolic acid, and D-leucine was observed in similar studies. In an experiment with D-methionine, Δ^1-pyrroline-2-carboxylic acid,[4] and D-amino acid oxidase, D-proline formation was shown to be accompanied by stoichiometric ammonia formation and D-methionine disappearance (Table I). In these experiments, the formation of α-keto-γ-methiolbutyric acid was demonstrated by preparation of the corresponding 2,4-dinitrophenylhydrazone and catalytic reduction of this derivative to methionine.[5] D-Methionine could be replaced by a number of other D-amino acids which are known to be oxidized by D-amino acid oxidase, but not by amino acids (*e.g.*, D-glutamic acid) which are not appreciably susceptible to oxidation. The formation of new amino acid was dependent upon the presence of flavin adenine dinucleotide (FAD). Formation of D-proline in reactions involving Δ^1-pyrroline-2-carboxylic acid, which may be regarded as a substituted imino acid, proceeded more rapidly, under the conditions employed, than did reactions with α-keto acids and ammonia.

Analogous studies have been carried out with purified L-amino acid oxidase[6]; for example, L-methionine was formed when L-leucine, L-amino acid oxidase, and α-keto-γ-methiolbutyric acid were incubated. The latter reaction may be represented as

$$\text{L-Leucine} + \text{Enzyme–FAD} + H_2O \rightleftharpoons \alpha\text{-Ketoisocaproate} + NH_3 + \text{Enzyme–FADH}_2$$

$$\text{Enzyme–FADH}_2 + NH_3 + \alpha\text{-Keto-}\gamma\text{-methiolbutyrate} \rightleftharpoons \text{L-Methionine} + \text{Enzyme–FAD} + H_2O$$

$$\text{Sum: L-Leucine} + \alpha\text{-Keto-}\gamma\text{-methiolbutyrate} \rightleftharpoons \text{L-Methionine} + \alpha\text{-Ketoisocaproate}$$

Although the over-all reaction is identical with a transamination reaction, it is clear that this reaction does not represent amino group transfer, but involves the intermediate participation of ammonia. Findings consistent with this interpretation are given in Table II. In these experiments, the purified oxidases were incubated with an amino acid substrate, its α-keto acid analog, and N^{15}-ammonia. The N^{15} content of the amino acid after incubation is recorded in the table[7]; isotope was not incorporated in control experiments in which the enzymes were inactivated by treatment with ethanol. These results conclusively demonstrate the reversibility of the amino acid oxidase reactions.

TABLE I

SYNTHESIS OF D-PROLINE BY D-AMINO ACID OXIDASE[a]

	D-Methionine utilized (μmoles)	D-Proline formed (μmoles)	NH_3 formed (μmoles)
Complete system[b]	0	0	0
Complete system	2.4	2.6	2.6
FAD omitted	0	0	0

[a] The reaction mixtures consisted of purified-D-amino acid oxidase preparation (10 mg.), D-methionine (25 micromoles), Δ^1-pyrroline-2-carboxylate (10 micromoles), and sodium pyrophosphate buffer of *p*H 8.3 (60 micromoles) in a final volume of 0.6 ml.; incubated at 37° for 180 minutes; gas phase, nitrogen. Disappearance of methionine was determined as described by K. V. Giri, A. N. Radhakrishnan and C. S. Vaidyanathan, *Anal. Chem.*, **24**, 1677 (1952); proline formation was determined by the procedure of W. Troll and J. Lindsley, *J. Biol. Chem.*, **215**, 655 (1955), as modified by A. Meister, A. N. Radhakrishnan and S. D. Buckley, *J. Biol. Chem.*, in press. [b] The enzyme was inactivated by treatment with 3 volumes of ethanol, or by heating at 100° for 5 minutes.

TABLE II

INCORPORATION OF N^{15}-AMMONIA INTO AMINO ACIDS CATALYZED BY AMINO ACID OXIDASES

Amino acid	Enzyme	Atom % excess N^{15} in amino acid
D-Alanine[a]	D-Oxidase	0.53
L-Leucine[b]	L-Oxidase	0.37

[a] Reaction mixture contained amino acid (50 micromoles), sodium pyruvate (25 micromoles), $N^{15}H_4NO_3$ (25 micromoles, 63 atom % excess $N^{15}H_4$), enzyme preparation and sodium pyrophosphate buffer of *p*H 8.3 (100 micromoles) in a final volume of 1.0 ml.; incubated at 37° for 120 minutes, under nitrogen. [b] Reaction mixture contained amino acid (10 micromoles), sodium α-ketoisocaproate (20 micromoles), $N^{15}H_4NO_3$ (20 micromoles, 63 atom excess $N^{15}H_4$), enzyme preparation (1.37 mg.), and tris-(hydroxymethyl)-aminomethane buffer of *p*H 7.2 (100 micromoles) in a final volume of 1.0 ml.; incubated at 37° for 180 minutes, under nitrogen. After incubation, the mixtures were made alkaline by addition of Na_2CO_3 and the free NH_3 was removed by exhaustive aeration. The α-amino nitrogen was subsequently released as NH_3 by treatment with the appropriate amino acid oxidase in air after readjustment of *p*H.

The configuration of the amino acids formed in these systems was established by determining the effect of purified L- and D-amino acid oxidases on the enzymatically synthesized amino acids. The α-keto acid analogs of amino acids which were not susceptible substrates were inactive in these systems; for example, α-ketoglutarate did not yield glutamic acid with either oxidase. L-Amino acids were not active in the D-amino acid oxidase system and vice versa.

Further studies on these systems may yield additional information concerning the mechanism of the amino acid oxidase reaction. It is possible that under appropriate physiological conditions, reactions of this type catalyzed by amino acid oxidases may represent a pathway alternative to transamination for interconversion of amino and keto acids.

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(8) Traveling Scholar of the J. N. Tata Endowment, India.

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(4) A. Meister, *J. Biol. Chem.*, **206**, 577 (1954).

(5) A. Meister and P. A. Abendschein, *Anal. Chem.*, **28**, 171 (1956).

(6) Prepared from snake venom as described by T. P. Singer and E. B. Kearney, *Arch. Biochem.*, **29**, 190 (1950).

THE REACTION OF TRIVALENT ORGANOPHOSPHORUS COMPOUNDS WITH POLYHALOMETHANES

Sir:

In view of the current interest in the reactions of tertiary phosphines,[1] of trialkyl phosphites,[2a] and

(1) R. N. Haszeldine and B. O. West, *J. Chem. Soc.*, 3631 (1956).

(2) (a) G. Kamai and L. P. Egoroda, *J. Gen. Chem. USSR.*, **16**, 1521 (1946); *C. A.*, **41**, 5439h (1947); (b) Z. L. Khisamova and G. Kamai, *J. Gen. Chem. USSR*, **20**, 1162 (1950); *C. A.*, **45**, 1531*d* (1951).