Studies on the Mechanism of Action of D-Amino Acid Oxidase

EVIDENCE FOR REMOVAL OF SUBSTRATE α-HYDROGEN AS A PROTON*

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SUMMARY

D-Amino acid oxidase catalyzes the oxidation of β-chloroalanine to chloropyruvate as well as its conversion to pyruvate, a nonoxidative reaction. The product composition depends on oxygen; only pyruvate is produced in 100% N₂, chloropyruvate is formed almost exclusively in 100% O₂, and mixtures result at intermediate levels. The rate of total keto acid production (pyruvate + chloropyruvate) is independent of oxygen, indicating the rate-determining formation of a common precursor of the two keto acids. Substitution of the α-hydrogen of β-chloroalanine by deuterium and tritium results in kinetic isotope effects which slow down chloroalanine conversion but do not differentially affect product distribution. A similar relationship holds for the deuterium solvent isotope effect with incubations conducted in D₂O. The substrate kinetic isotope effects indicate that the dissociation of the α-C—H bond of the substrate is rate-determining or at least partially rate-determining in the formation of the common intermediate. Preliminary experiments indicate that D-amino acid oxidase also produces both pyruvate and chloropyruvate from L-chloroalanine. These results suggest that the conversion of chloroalanine to pyruvate and chloropyruvate by D-amino acid oxidase involves a common intermediate in which the α-hydrogen of the substrate has been removed as a proton. It is very probable that the oxidation of other amino acid substrates involves similar intermediates. The intermediate could be a carbanion or an adduct derived from a substrate carbanion and the flavin coenzyme.

D-Amino acid oxidase does not catalyze the exchange of the α-hydrogen of chloroalanine and serine with solvent protons under anaerobic conditions. When [α-³H]proline, pyruvate, and NH₄⁺ were incubated anaerobically with the enzyme, no ³H was observed in the alanine formed in such incubations. This indicates that the proton released from the substrate is rapidly released from FADH₂.

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The kinetics of the D- and L-amino acid oxidase reactions have been extensively studied and the kinetic interrelationships between various spectroscopically identifiable intermediates are well worked out (1–3). However, the chemistry involved in the oxidative deamination is not clear, nor are the exact structures of the spectroscopically identifiable species known. Two general chemical mechanisms can be envisaged. Electrons could be transferred from the substrate to the coenzyme at a time, which would give rise to radical intermediates. Such mechanisms have been proposed both for D- and L-amino acid oxidase, although conclusive evidence for the occurrence of radical intermediates is lacking (1, 2). Alternatively, ionic mechanisms can occur in which the transfer of electrons occurs either through a hydride ion transfer from substrate to coenzyme, or by direct electron transfer coupled with proton transfer (4, 5). For an ionic mechanism an α-carbanion derived from the amino acid would be one attractive intermediate. Such a carbanion has been suggested by Neims, De Luca, and Hellerman (6) based on substituent effects on reaction rates with substituted aromatic amino acids. More recently, a mechanism has been proposed by Brown and Hamilton (5), based on model studies, which involves the formation of a carbanion-like intermediate after addition of the amino group of the amino acid to the Cα position of FAD. We have carried out experiments with D-amino acid oxidase, and in a preliminary way with L-amino acid oxidase, designed to examine further the possible intermediate involvement of α-carbanions or carbanion-like structures in the D- and L-amino acid oxidase reactions. β-Chloroalanine appeared to us to be a substrate which could provide information concerning the nature of the enzymatically produced intermediates. Its special usefulness derives from the fact that, if the enzyme catalyzes the abstraction of the α-hydrogen as a proton, chloride ion would readily be eliminated from the β position of the substrate.

The keto acid product would then be pyruvate. Pyruvate formation, therefore, would provide evidence for enzymatic α-proton abstraction. The possible reactions of chloroalanine, including its oxidative deamination are shown in Scheme 1.

Chloropyruvate would be the expected product formed by the amino acid oxidase, whereas pyruvate would be derived from the breakdown of the enzymically produced carbanion in a non-oxidative process.
Experimental Procedure

Materials

Crystalline hog kidney D-amino acid oxidase was obtained from Sigma and was homogeneous by disc gel electrophoresis (7, 8). It had a specific activity of about 5 μmol per min per mg when measured with D-alanine as substrate as indicated in Table I. Purified L-amino acid oxidase from Crotalus adamanteus was obtained from Nutritional Biochemicals as was lyophilized catalase. L-[3H]Glycine (100 μCi) and L-[14C]glycine, and H2O were obtained from New England Nuclear. D2O was purchased from Bio-Rad, Richmond, California. D,L-[α-3H]Proline was obtained from Mr. Gary Rudnick of this laboratory. D,L-β-Chloroalanine-HCl were purchased from Cycle Chemical Company, Los Angeles, Calif.

Hydroxypyruvate was obtained from Nutritional Biochemicals.

Methods

Chemical Synthesis

Chloropyruvate—Crude chloropyruvate acid was synthesized by reaction of sulfonyl glycine with freshly distilled pyruvic acid (9). Sulfuryl chloride, 3.8 ml (47 mmoles), was added dropwise to 3.2 ml (46 mmoles) of freshly distilled pyruvic acid and allowed to react with stirring for 72 hours at room temperature. The yellow semisolid paste was then subjected to vacuum distillation to obtain pure chloropyruvic acid. When the solid mixture was heated, liquid distilled over at 92° at 3 mm Hg pressure (pyruvic acid distills at 40° at 3 mm Hg), and crystallized in the condenser, as reported by Nair and Busch (10). The crystals were dried over P2O5 for 12 hours and gave a melting point of 82–84° (reported value, 82–84° (10)). An infrared spectrum of the solid in KBr pellet displayed a peak at 1750 cm−1, consistent with an α-keto acid.

D,L-α-[3H]Serine-HCl—D,L-Serine specifically tritiated in the α position was synthesized from glycine by condensation with formaldehyde (11). It was observed in preliminary experiments starting with glycine in H2O that only limited exchange of the glycine methylene protons occurred with water prior to condensation. Thus α-[3H]glycine was used as starting material. To 225 mg of α-[3H]glycine (3,000 μCi, 990 μCi) in a 50-ml round-bottom flask were added 24 ml of 0.5 M sodium carbonate (12,000 μmoles), 300 μl of 0.1 M copper sulfate (30 μmoles), and 2 ml of 12 M formaldehyde (24,000 μmoles). After refluxing for 1 hour, the solution was cooled and filtered through a sintered glass funnel to remove copper hydroxide deposits. The solution was acidified to pH 6 with glacial acetic acid and freed of carbon dioxide on a rotary evaporator for 3 min. The solution was applied to a 3 × 14 cm column of Dowex 50-H+, and the column was washed with 400 ml of water until no more radioactivity eluted (no ninhydrin-positive material eluted in the water wash). Amino acids were eluted in bulk with 2 N ammonium hydroxide and the eluate was concentrated to 5 ml on a rotary evaporator. Eight volumes of 95% ethanol were added and the opalescent solution was chilled overnight. The resulting crystals were centrifuged at 2,000 rpm for 2 min in a clinical centrifuge and the supernatant was discarded. The crystals were dissolved in 6 ml of water, applied to a 1.5 × 82 cm column of Dowex 50-H+, and eluted with 1 N HCl. The first radioactive peak (of two eluted) was subjected to electrophoresis with authentic serine as marker on paper electrophoresis at pH 1.9 (Whatman No. 53M bonded paper, 71 volts per cm for 26 min) and this material contained only one ninhydrin-positive spot corresponding to serine. The second radioactive peak from the Dowex column was unreacted glycine. The serine-containing fractions were evaporated to dryness and the crystalline residue was desiccated over P2O5 and NaOH pellets. The yield (150 mg; 1,070 μmoles) was 33% and the material had a specific activity of 70,000 cpm of H per μ mole (30% efficiency).

That the serine was labeled exclusively in the α position was tested by subjecting a sample to periodate oxidation followed by distilling the formaldehyde and trapping the distilled aldehyde as the dimedon derivative (12). Authentic β-[3H]Serine served as a control in this procedure and [14C]formaldehyde recovered in 65% yield. The dimedon derivative from the [α-3H]serine oxidation had 38 cpm of 3H over background (20 cpm) whereas a 65% yield of [14C]formaldehyde would have been 22,000 cpm (30% efficiency).

D,L-α-[3H]Chloroalanine HCl—The synthesis of chloroalanine from serine reported by Fischer and Raske (13) and also by Groenstein and Winitz (14) depends on the chlorination of serine methyl ester in freshly distilled acetyl chloride with PCl5. In 12 attempts we were unsuccessful in reproducing this step and subsequently used a different solvent for the chlorination (15). For a successful synthesis, both the methyl ester and the solvent chloroform must be scrupulously dry. D,L-[α-3H]Serine-HCl (50 mg) was mixed with 100 mg of nonlabeled DL-serine-HCl and bottom flask were added 24 ml of 0.5 M sodium carbonate (12,000 μmoles), 300 μl of 0.1 M copper sulfate (30 μmoles), and 2 ml of 12 M formaldehyde (24,000 μmoles). After refluxing for 1 hour, the solution was cooled and filtered through a sintered glass funnel to remove copper hydroxide deposits. The solution was acidified to pH 6 with glacial acetic acid and freed of carbon dioxide on a rotary evaporator for 3 min. The solution was applied to a 3 × 14 cm column of Dowex 50-H+, and the column was washed with 400 ml of water until no more radioactivity eluted (no ninhydrin-positive material eluted in the water wash). Amino acids were eluted in bulk with 2 N ammonium hydroxide and the eluate was concentrated to 5 ml on a rotary evaporator. Eight volumes of 95% ethanol were added and the opalescent solution was chilled overnight. The resulting crystals were centrifuged at 2,000 rpm for 2 min in a clinical centrifuge and the supernatant was discarded. The crystals were dissolved in 6 ml of water, applied to a 1.5 × 82 cm column of Dowex 50-H+, and eluted with 1 N HCl. The first radioactive peak (of two eluted) was subjected to electrophoresis with authentic serine as marker on paper electrophoresis at pH 1.9 (Whatman No. 53M paper, 71 volts per cm for 26 min) and this material contained only one ninhydrin-positive spot corresponding to serine. The second radioactive peak from the Dowex column was unreacted glycine. The serine-containing fractions were evaporated to dryness and the crystalline residue was desiccated over P2O5 and NaOH pellets. The yield (150 mg; 1,070 μmoles) was 33% and the material had a specific activity of 70,000 cpm of H per μ mole (30% efficiency).

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dissolved in 6 ml of methanol that had been dried with a Linde 4A molecular sieve. HCl gas, dried by passage through concentrated H₂SO₄, was bubbled through the methanol for 30 min after which the solution was refluxed another 30 min. The solution was evaporated to dryness, and the solid was suspended in dry benzene and evaporated to dryness twice. The crystals were dissolved in 2 ml of dry methanol and 30 ml of dry ether were added slowly at room temperature. The opalescent solution was chilled for 2 hours and the crystals were collected on a sintered glass funnel and washed with ether. The yield was 146 mg of DL-[α-²H]serine methyl ester-HCl (90%), melting point 129–131° (reported value, 133° (16)).

The serine methyl ester was chlorinated with PC₁₅ in dry chloroform. Chloroform was freed of ethylene by passage through an alumina (Woelm) column and stored over a Linde 4A molecular sieve for not more than 2 hours before use. To 5 ml of the dry chloroform in a two-neck 50-ml round bottom flask were added 210 mg of phosphorous pentachloride, which dissolved within 30 min with stirring at room temperature. The flask, containing a CaCl₂ drying tube in one neck and a ground glass stopper in the other, was chilled in an ice bath and 140 mg of dried, pulverized DL-[α-²H]serine methyl ester-HCl were added in small aliquots over a 30 min period while the solution was stirred at 0°. Then the flask was stirred at room temperature for 2 hours until a clear solution developed. After the flask was cooled overnight and petroleum ether added, crystals of DL-[α-²H]β-chloroalanine methyl ester-HCl were collected and washed with petroleum ether. The yield was 105 mg (66%). The solid ester was added to 4 ml of 6 HCl and refluxed for 1 hour. The acid was removed by rotary evaporation to dryness, the residue was dissolved in water and evaporated to dryness twice, and the procedure was repeated twice from benzene. The solid material was dissolved in 1 ml of dry methanol and ether was added to opalescence. After chilling for 30 min, the crystals were collected by filtration and washed with ether. The yield was 75 mg (51%) (over-all yield from serine, 32%) of DL-[α-²H]chloroalanine-HCl. The product displayed one ninhydrin-positive spot on paper electrophoresis and paper chromatography as described for the tritiated material.

Assays

Determination of Keto Acids Total α-keto acids were estimated as the respective 2,4-dinitrophenylhydrazine derivatives (18). After 10 min the developed color was read in a Klett-Summerson colorimeter fitted with a 54 filter, then scanned from 650 to 400 nm in a Unicam SP 800 recording spectrophotometer. When estimated by this protocol, pyruvic acid gave a value of 900 ± 20 Klett units per μmole; chloropyruvate, 1200 ± 20 Klett units per μmole; and hydroxychloroacetate, a value of 800 ± 20 Klett units per μmole.

Pyruvic acid was estimated spectrally by conversion to its thiosemicarbazone. Fortuitously, either chloropyruvic acid does not so readily form a thiosemicarbazone or it has a much lower extinction coefficient. Aliquots of pyruvic acid, chloropyruvic acid, or enzyme incubations were added to 1 ml of a 100 mM thiosemicarbazide solution in 0.1 M sodium acetate (pH 5.4). Formation of derivatives was complete within 10 min. Thiosemicarbazone spectra were recorded, against a blank solution containing reagent and water instead of sample, in a Unicam SP 800 recording spectrophotometer from 350 to 250 nm. The thiosemicarbazone of pyruvic acid displays a λmax at 287 nm with an E₅₀₀ of 14,000 liters cm⁻¹ mole⁻¹. By comparison, the thiosemicarbazone from chloropyruvate gave only slight absorption with an E₅₀₀ of about 100 liters cm⁻¹ mole⁻¹.

Chloropyruvate content in enzymatic incubations was quantitated after separate analysis of total keto acid 2,4-dinitrophenylhydrazone and of pyruvate thiosemicarbazone. In incubations in which the spectra of the 2,4-dinitrophenylhydrazones indicated detectable pyruvate contributions, the thiosemicarbazone absorbance was assumed to represent only pyruvate. Subtraction of the pyruvate content (800 Klett units per μmole) from the total keto acid content in the DNP assay leaves the contribution due to chloropyruvate DNP (1200 Klett units per μmole).

Rates of Enzymic Reactions—In several enzymic experiments, incubations were flushed with mixtures of oxygen and nitrogen.

1 The abbreviations used are: DNP, 2,4-dinitrophenylhydrazone; E-FAD, enzyme-coenzyme complex containing oxidized flavin; E-FADH₂, enzyme-coenzyme complex containing fully reduced flavin.
This was usually accomplished by conducting incubations in vials of cuvettes stoppered with closely fitting rubber serum bottle caps. In some experiments, the caps were sealed with Apiezon grease, but this precaution proved unnecessary. The rubber caps were punctured with two needles: one for inlet and the other for outlet of gas. Gas mixtures were passed into incubations for at least 5 min prior to the start of incubations. The gas inflow was bubbled into the solutions gently for 3 min, then the tip of the inlet needle was maintained over the surface of the solution for the last 2 min of the preincubation and throughout incubations during which a slight positive gas pressure was maintained. The outlet needle was always maintained above the surface of the solutions. Reactions were started by addition of either enzyme or substrate from solutions, gassed with the same gas composition as in incubations, passed in through the inlet needle.

Aliquots were removed from incubations by disconnecting the gas inlet tubing from the needle, pushing the needle below the surface of the solution, and withdrawing aliquots into a 250-μl gas-tight Hamilton syringe. When the aliquot was in the syringe, the syringe was detached from the needle, and the aliquot was used either for total keto acid assay or thiosemicarbazone or both, after addition to an equal volume of redistilled methanol to stop the enzymatic reaction. The gas inlet tubing was immediately reattached to the needle and the holdup volume in the needle was gently forced back into the incubation solution.

In experiments in which aliquots were not frequently removed and more anaerobic conditions were sought, incubations were conducted in vessels containing center wells and side arms, fitted with a well greased stopcock. Reaction components were placed in appropriate arms and the entire vessel was alternately evacuated then filled with argon for five cycles. Incubations proceeded under argon. Results with those more stringent anaerobic conditions mirrored those from the above sampling procedure.

Silicic Acid Chromatography Reaction products from enzymatic incubations with [3-14C]chloroalanine were identified by decarboxylation with neutral hydrogen peroxide and the resulting 14C chloroacetic and 14C acetate were separated by silicic acid column chromatography.

Each incubation contained (in 3.0 ml): 50 mM NaPi (pH 8.5); 160 μmoles (10,000 cpm per μmole) of DL-[3-14C]chloroalanine, freshly dissolved and neutralized, 150 μg of catalase, and 250 μg of d-amino acid oxidase added to start the incubations. One incubation proceeded under 100% O₂ atmosphere, the other under 100% N₂ atmosphere. Incubations were for 30 min at which time 50-μl aliquots were removed and assayed for total keto acid, and their spectra were recorded. The spectra indicated only pyruvate in the anaerobic reaction and only chloropyruvate in the aerobic reaction. Incubations were terminated by addition of 200 μg of Dowex 50-H⁺ and then the resulting suspensions were added to 0.5 × 6 cm columns of Dowex 50-H⁺ in H₂O. After samples were applied, the columns were washed with 3 ml of H₂O to remove keto acids from the column without removing proteins and unreacted [14C]chloroalanine. The Dowex 50-H⁺ effluents (6 ml) were assayed for keto acid as above and shown to contain 80% of the keto acids originally present at the end of the incubation. Then the pH was adjusted to 7 with 2 N KOH and 0.8 ml of 30% hydrogen peroxide was added. Decarboxylation of the α-keto acids was allowed to proceed at room temperature for 1 hour. Lyophilized catalase, in about 0.5-μg aliquots, was added until O₂ evolution had ceased. The pH of the solutions was adjusted to 10.5 with 2 N KOH and the samples were concentrated to dryness on the rotary evaporator. The samples were stored dry for ~4 hours while two silicic acid columns were prepared in dry chloroform according to the method of Varner (19). Eight grams of oven-dried (140° overnight) silicic acid were used for each column. The two samples were then dissolved in 0.4 ml of 0.5 N H₂SO₄ and 10 μl of 36% H₂SO₄. Chloroacetic acid standard (200 μmoles) was added to the sample from the N₂ incubation and 200 μm of acetate acid standard were added to that from the O₂ incubation. Each sample was mixed with 1 g of silicic acid and then slurried in chloroform and added to each column. The columns were washed with 10 ml of chloroform and then acids were eluted with 4% 1-butanol in chloroform. Fractions of 1.5 ml were collected at a flow rate of 4.5 ml per hour. The two columns were developed at the same time and same flow rates with the same fraction collector. Column fractions contained 0.5 ml of H₂O added to each fraction before development of the column. Aliquots (50 μl) of the water phase only of each fraction were counted in 10 ml of Bray’s scintillation fluid (20). Then, each fraction, both H₂O and CHCl₃ layer, was added to a 5-ml beaker, containing 1.5 ml of H₂O and titrated to pH 7 with 0.1 N NaOH. This procedure located the standard acetate and chloroacetic acids. Aliquots of the H₂O layer were again counted and found to contain about 2.5 times as much 14C after titration as before. With this fact, it was calculated that recoveries of 14C material from the column were greater than 90%.

RESULTS
Products Derived from Chloroalanine in Presence of d-Amino Acid Oxidase

Anaerobic Reaction—When DL- or n-chloroalanine was incubated with d-amino acid oxidase in an atmosphere of 100% N₂, formation of a keto acid product was observed. Spectrum 1 in Fig. 1A represents the absorption spectrum of the 2,4-dinitrophenylhydrazones derived from the keto acid product. This spectrum is to be compared with Curves 1 and 2 in Fig. 1B which are, respectively, the 2,4-dinitrophenylhydrazones of authentic chloropyruvate and pyruvate. The enzymatic product under anaerobic conditions appears to be pyruvate, not chloropyruvate. When other amino acids were tested as substrates (alanine, serine, leucine), no keto acid product was detected under the above reaction conditions (see, for instance, Curve 3 in Fig. 4).

To confirm the unusual finding of pyruvate as the anaerobic reaction product from chloroalanine, the spectrum of the keto acid thiosemicarbazone was examined (Curve 1, Fig. 2A). The spectra of pyruvate and chloropyruvate thiosemicarbazones are shown for comparison in Fig. 2B. The thiosemicarbazone of the enzymatic keto acid product under anaerobic conditions with chloroalanine has a spectrum identical with that of pyruvate. Authentic pyruvate yields a thiosemicarbazone with a λmax at 287 nm and a molar extinction of 14,000 liters mol⁻¹ cm⁻¹. Chloropyruvate, under the same conditions, produces a thiosemicarbazone with an extinction coefficient of only 100 at 287 nm, less than 1% of the value for the pyruvate derivative. In the anaerobic incubations the amount of pyruvate detected by thiosemicarbazone formation accounted for all the keto acid.
2,4-dinitrophenylhydrazone produced. Thus, pyruvate appears to be the sole product produced from chloroalanine in the absence of oxygen.

To characterize further the pyruvate formed, the 2,4-dinitrophenylhydrazone that formed after incubation under 100% N₂ was isolated as a crystalline solid in 80% yield. After recrystallization, the solid melted at 217-218°. The DNP of authentic pyruvate had a melting point of 218-219°. No depression in melting point was observed in a mixed melting point determination. Pyruvic acid was also subsequently identified by conversion to acetic acid and chromatography on a silicic acid column (see Fig. 5, Panel B).

The stoichiometry of the anaerobic reaction was next investigated. Although the chloroalanine was free of detectable alanine contamination by electrophoretic and paper chromatographic criteria, it was imperative to demonstrate that the anaerobically produced pyruvate was derived from chloroalanine. This fact is indicated in Fig. 3. In the enzymatic reaction chloroalanine disappearance is mirrored by pyruvate appearance.

**Fig. 2.** A, spectra of keto acid thiosemicarbazones from incubations with chloroalanine. Each incubation contained the components listed in the legend to Fig. 1A. At the end of a 5-min incubation, aliquots (100 μl for Curves 1 and 3; 300 μl for Curve 2) were removed, added to 1 ml of 100 mM thiosemicarbazide in 100 mM sodium acetate (pH 5.4) and allowed to react for 10 min. Spectra were recorded against a blank from an incubation lacking enzyme. Curve 1, incubation under 100% N₂; Curve 2, incubation under 8% O₂; Curve 3, incubation under 100% O₂. B, spectra of thiosemicarbazones of pyruvate and chloropyruvate. Aliquots of pyruvate (0.30 μmole) and of chloropyruvate (0.30 μmole) were added to thiosemicarbazide reagent and spectra recorded as in A. Curve 1, chloropyruvate derivative; Curve 2, pyruvate derivative.

**Fig. 3 (left).** Stoichiometry of p-chloroalanine reaction with d-amino acid oxidase under anaerobic conditions. The incubation mixture contained (in 1.5 ml): 20 mM sodium pyrophosphate (pH 8.5); 40 mM DL-chloroalanine; 50 μg of catalase; and 100 μg of d-amino acid oxidase (dialyzed overnight against 50 mM sodium phosphate, pH 6.5). The incubation was conducted in a N₂ atmosphere and was initiated by addition of chloroalanine. Aliquots were removed at intervals and assayed for keto acid or for amino acid by ninhydrin assay (21). Spectra of the DNP of the keto acid product indicated that pyruvate was the only product. Since only the 1 isomer of chloroalanine is a substrate the initial value of micromoles of 1 isomer has been subtracted from the values for micromoles of total amino acid remaining, obtained with the ninhydrin assay. There is no nonenzymatic breakdown of chloroalanine under the assay conditions during the incubation.

**Fig. 4 (right).** Anaerobic incubation of alanine or chloroalanine. Each incubation mixture contained (in 1.0 ml): 20 mM sodium pyrophosphate (pH 8.5); 40 mM d-alanine or 15 mM d-chloroalanine; 50 μg of catalase; and 50 μg of d-amino acid oxidase added last. Aliquots of 100 μl were withdrawn at intervals and assayed for pyruvate and for total keto acids as described under "Methods." Pyruvate was the only product. Both incubations were under N₂ atmosphere at 25°. Curve 1, p-chloroalanine as substrate; Curve 2, d-alanine as substrate. Oxygen was admitted to the system as indicated by the arrows.
Under identical reaction conditions with alanine as enzymatic substrate, no pyruvate was detected. Also, there is no non-enzymatic breakdown of chloroalanine to pyruvate during the time of the experiment.

A typical time course for anaerobic generation of pyruvate from chloroalanine is depicted in Fig. 4, Curve 1. The production of pyruvate is initially linear, for about 10 min, then falls off and essentially ceases at about 20 min. This behavior was reproduced in many incubations. Cessation of reaction does not reflect exhaustion of substrate, for only 6 of the 15 pmoles with the oxidized form (E’FAD) or the reduced form detected.

In the absence of oxygen, it should be emphasized that in the enzymatic breakdown of chloroalanine to pyruvate during the reaction, as indicated, induced further keto acid formation. Curve 2 of Fig. 4 confirms the hypothesis that anaerobic conditions prevailed in these incubations. When D-alanine was employed as substrate, no keto acid product was detected until oxygen was admitted to the system. This stresses the unique ability of chloroalanine to function as a substrate for d-amino acid oxidase in the absence of oxygen. It should be emphasized that in the experiment of Curve 2 in Fig. 1, the stoichiometric reaction of chloroalanine is depicted in Fig. 4, Curve 1. The production of pyruvate is initially linear, for about 10 min, then falls off and essentially ceases at about 20 min. This behavior was reproduced in many incubations. Cessation of reaction does not reflect exhaustion of substrate, for only 6 of the 15 pmoles with the oxidized form (E’FAD) or the reduced form detected.

The question then arises as to whether chloroalanine reacts with the oxidized form (E–FAD) or the reduced form (E–FADH2) of the enzyme-coenzyme complex. To test this, the fully reduced E–FADH2 was generated and maintained anaerobically in two ways: (a) by addition of 2 μmoles of sodium dithionite to the enzyme solution prior to addition of chloroalanine or (b) by addition of 2 μmoles of alanine prior to addition of chloroalanine, both under the incubation conditions of Fig. 1. In each case, no keto acid product was detected during the 20-min incubation. Subsequent addition of oxygen to the system generated keto acid product. This indicates that chloroalanine reacts with the oxidized but not the reduced form of the coenzyme-enzyme complex. It might be argued that dithionite treatment, although leading to an enzyme-coenzyme complex which is fully reduced by spectral analysis (1), may yield a chemically inactive E–FADH2. However, we demonstrated that the dithionite-reduced coenzyme-enzyme complex was capable of catalyzing the reverse reaction in an experiment analogous to those of Radhakrishnan and Meister (22). To 2 μmoles of enzyme-FAD, under anaerobic conditions (see Table V), 3 μmoles of dithionite were added followed by 20 μmoles each of pyruvate and ammonia. After 40 min at room temperature, reaction products were analyzed by paper electrophoresis. A total of 1.0 μmole of alanine was found. This shows the catalytic competence of the dithionite-reduced enzyme and proves that conversion of chloroalanine to pyruvate is catalyzed only by enzyme-FAD.

**Incubations in 100% O2**—The above data have indicated that anaerobic incubations of D-alanine oxidase with β-chloroalanine yield pyruvate exclusively. When similar incubations were conducted with 100% O2 as the gas phase, the expected keto acid product, chloropyruvate, was formed almost exclusively. Curve 2 of Fig. 1A indicates that the spectrum of the keto acid-DNP from 100% O2 incubations looks identical with the spectrum of the authentic chloropyruvate derivative, Curve 1 of Fig. 1B. Confirmation of this point was obtained when the enzymatic product was assayed for its ability to form a thiosemicarbazone. A duplicate aliquot of the keto acid product that gave the DNP spectrum noted above, produced the spectrum of Curve 3 in Fig. 2A. Almost no thiosemicarbazone is formed and the pyruvate content of the enzymatic keto acid product in 100% O2 is less than 5% by these criteria.

To characterize further chloropyruvate, we attempted to crystallize the DNP derivative formed in the above enzymatic reaction. Despite some effort no crystalline 2,4-dinitrophenylhydrazone of either the enzymatic reaction product from 100% O2 incubations or of authentic chloropyruvate (or authentic bromopyruvate) could be obtained. Further, on standing overnight in acidic solution, the spectrum of the chloropyruvate-DNP changed to a spectrum analogous to that of pyruvate-DNP, indicating degeneration of the derivative.

The additional identification of the aerobic reaction product as chloropyruvate was then obtained by decarboxylation with neutral hydrogen peroxide (23), followed by isolation of the resulting chloroacetate. Duplicate incubations were carried out with [β-14C]chloroalanine: one in 100% O2 atmosphere; the other in 100% N2 atmosphere (to produce pyruvate). In 30 min, 23 μmoles of chloropyruvate and 20 μmoles of pyruvate were generated, respectively, from 80 μmoles of substrate. After decarboxylation, each acid was chromatographed on identical silicic acid columns with the results shown in Fig. 5. In Fig. 5A it is observed that 75% of the labeled acid is [14C]chloroacetate with the remainder as [14C]acetate. Thus, at least...
75% of the keto acid formed enzymatically in 100% O2 atmosphere is chloropyruvate. This represents a minimal figure since chloropyruvate may decompose partially before the hydrogen peroxide treatment. In Fig. 5B it is observed that only acetate is recovered from the anaerobic reaction mixture, consistent with pyruvate formation exclusively under those conditions.

Effect of Oxygen Level on Product Composition and Maximal Velocity

Since different keto acids are produced enzymatically from β-chloroalanine at extremes of oxygen concentration, it might be anticipated that at intermediate oxygen levels, mixtures of the two products would be formed. That this is the case is clear from Curve B in Fig. 1A in which the spectrum of the DNP derived from the product in 8% O2 appears to be a composite of the two extreme CURVES of Fig. 1B. Fig. 2D confirms that the pyruvate thiosemicarbazone produced in 8% O2 is less than that formed in 100% N2, but greater than that formed in 100% O2.

Since the contribution of chloropyruvate to thiosemicarbazone absorption can be essentially neglected, this assay when coupled with the total keto acid 2,4-dinitrophenylhydrazone assay allows quantitation of the amounts of pyruvate and chloropyruvate present in any enzymatic product mixture. It is found that at 20% O2 about 70% of the keto acid product is chloropyruvate and 30% is pyruvate.

To explore more fully the singular phenomenon of two keto acid products from β-chloroalanine in d-amino acid oxidase incubations, the percent oxygen in the gas phase was varied from 0 to 100% in binary O2-N2 gas mixtures. The results of such incubations are shown in Fig. 6. Two facts emerge from the data in this figure. Most striking is the fact that over the whole range of O2 concentrations the total keto acid product formed remained constant. These incubations were performed at saturating substrate levels (30 times the Km) and thus reflect a constant maximal velocity independent of O2. This is in contrast to other amino acid substrates in which Vmax depends on oxygen concentration and only a stoichiometric reaction occurs at 0% O2 (1, 24). It is also shown in Fig. 6 that with increasing oxygen concentration the percentage of chloropyruvate, the expected keto acid product also increases.

Comparison of Chloroalanine and Alanine as Substrates for d-Amino Acid Oxidase

Since chloroalanine is so far unique among known substrates for hog kidney d-amino acid oxidase, it was germane to compare some of its kinetic properties with that of a "normal" substrate such as alanine. Dixon and Kleppe (25) reported a Km for d-alanine of about 2 mm. The d isomer of chloroalanine displays normal hyperbolic saturation kinetics and has a Km of 0.5 mm in 20% O2 and in 100% N2. Thus, by this criterion, chloroalanine is an effective substrate for the enzyme. With regard to maximal velocities, alanine and chloroalanine react at about the same velocity with 20% O2 in the gas phase as seen in Table I. It has been observed that alanine and other amino acids display ping-pong kinetics when O2 is the second substrate (1, 24). In Table 1 alanine shows a Vmax dependent on O2 (7 pm per min per mg at 100% O2 versus 5 pm per min per mg at 20% O2). As noted above chloroalanine shows a Vmax independent of O2 (about 5 pm per min per mg). These velocity data indicate that the dismutation of chloroalanine to the two possible keto acid products is as fast as reaction of a good normal substrate and thus may reflect a central catalytic capacity of the enzyme and bear upon the normal mode of reaction of amino acid substrates with this enzyme.

The fact that chloroalanine reacts with the same maximal rate in the presence and absence of oxygen suggests that the formation of some intermediate occurs the breakdown of which is not rate-determining; that is, even when insufficient oxygen is present, its breakdown by a pathway leading to pyruvate is fast enough to react with every molecule of intermediate formed.

Comparison of alanine and chloroalanine as substrates for d-amino acid oxidase

Each incubation mixture contained: 20 mM NaPPi (pH 8.5), 50 µg of catalase; alanine or chloroalanine as indicated, 15 mm in d-isomer; and 100 µg of d-amino acid oxidase added last, after a 5-min prior incubation of the other components in the indicated oxygen atmosphere. Products were assayed for total keto acid and for pyruvate as described under "Experimental Procedure." Volume, 2.0 ml; temperature, 25°. Vo represents total keto acid formation.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Vmax</th>
<th>Product</th>
<th>O2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µm/min/mg</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>D-Alanine</td>
<td>5.0</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>D-Alanine</td>
<td>7.0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>D-Alanine</td>
<td>9.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L-Chloroalanine</td>
<td>5.5</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>D-Chloroalanine</td>
<td>5.0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>D-Chloroalanine</td>
<td>5.4</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>
Table II

Proton Abstraction by D-Amino Acid Oxidase
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Each incubation contained in 1.0 ml at 25°C: 50 mM sodium pyrophosphate (pH 8.5), 50 μg of catalase; and 500 μg of D-amino acid oxidase. In incubations with DL-α-[2H]Serine, 40 nmol were used (90,000 cpm of 2H per nmol); 40 nmol of DL-α-[2H]chloroalanine were also used (18,000 cpm of 2H per nmol). Aliquots (200 μl) were withdrawn after 30 sec and 2, 5, 10, and 15 min, and quenched with an equal volume of methanol. One-half of the quenched solution was used for keto acid assay (in chloroalanine experiments, both pyruvate and chloropyruvate were determined). The other half of the 50% methanolic solution was applied to a 3-cm column of Dowex 50-H+ in a disposable Pasteur pipette. The column was then washed with 2 ml of water and the eluate was collected directly into a vial containing 10 ml of scintillation fluid. It was separately determined that recovery of authentic 2H2O from these columns was greater than 98%. The amount of 2H-amino acid converted to keto acid was directly calculated from the amount of 2H2O eluted from Dowex. Control tubes lacking enzyme released no 2H on elution of the Dowex columns with 2 ml of water.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>O2</th>
<th>Keto acida</th>
<th>% 2H released</th>
<th>&amp;H/ORH</th>
</tr>
</thead>
<tbody>
<tr>
<td>2H-Chloroalanine</td>
<td>0</td>
<td>16.3</td>
<td>1.6</td>
<td>10.2</td>
</tr>
<tr>
<td>2H-Chloroalanine</td>
<td>20</td>
<td>13.7</td>
<td>1.4</td>
<td>10.2</td>
</tr>
<tr>
<td>2H-Chloroalanine</td>
<td>100</td>
<td>14.3</td>
<td>1.4</td>
<td>10.2</td>
</tr>
<tr>
<td>2H-Serine</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2H-Serine</td>
<td>20</td>
<td>5.3</td>
<td>1.0</td>
<td>5.3</td>
</tr>
<tr>
<td>2H-Serine</td>
<td>100</td>
<td>7.6</td>
<td>1.6</td>
<td>4.8</td>
</tr>
</tbody>
</table>

* Measured as total keto acid produced.
3 Measured as 2H released into H2O.

Isotope and Exchange Reactions; Effect of α-2H and α-2H Substrates

To determine if the breaking of the amino acid α-C-H bond is rate-determining in the reaction catalyzed by D-amino acid oxidase, we prepared [α-2H]- and [α-2H]chloroalanine (and serine). The results of enzymatic incubations with the tritiated substrates are presented in Table II: results with the deuterated substrates are shown in Table III. It is clear from Table II that substitution of the 2H-α by 2H in the case of chloroalanine results in a selection against tritium of about 10. It is noted that this tritium selection is essentially constant even when the nature of the keto acid product changes from wholly pyruvate to almost exclusively chloropyruvate. This tritium selection seen by [α-2H]chloroalanine suggests that transfer of the α-hydrogen is rate-determining or at least partially rate-determining. That the discrimination against α-H does not change with changing product composition indicates that there is no differential selection against the α-2H in the two reaction pathways.

This selection against the α-tritium is a feature of the reaction sequence with other amino acids since it is also seen with serine. [α-2H]Serine, a close structural analogue of chloroalanine, experienced an α-2H selection of about 5 at both 20% O2 and at 100% O2 at which the Vmax was increased by 50%. Hydroxy-

pyruvate was the sole product in each instance. Under anaerobic conditions, neither keto acid product nor release of 2H into water was detected with serine.
TABLE IV
Effect of $^3$H$_2$O on reaction with [α-$^3$H]- and [α-$^3$H]-chloroalanine

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Substrate</th>
<th>Solvent</th>
<th>$^{3}O_{2}$</th>
<th>Keto acid</th>
<th>Pyruvate</th>
<th>Chloropyruvate</th>
<th>pH/$^3$H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>[α-$^3$H]-Chloroalanine</td>
<td>$^3$H$_2$O</td>
<td>0.95</td>
<td>23</td>
<td>77</td>
<td>2.02</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>[α-$^3$H]-Chloroalanine</td>
<td>$^3$H$_2$O</td>
<td>0.41</td>
<td>23</td>
<td>77</td>
<td>1.98</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>[α-$^3$H]-Chloroalanine</td>
<td>$^3$H$_2$O</td>
<td>0.48</td>
<td>22</td>
<td>78</td>
<td>2.08</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>[α-$^3$H]-Chloroalanine</td>
<td>$^3$H$_2$O</td>
<td>0.50</td>
<td>100</td>
<td>0</td>
<td>3.13</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>[α-$^3$H]-Chloroalanine</td>
<td>$^3$H$_2$O</td>
<td>0.38</td>
<td>20</td>
<td>80</td>
<td>1.89</td>
<td></td>
</tr>
</tbody>
</table>

With a deuterium kinetic isotope effect of 1.9, the Swain equation predicts a tritium kinetic isotope effect of 2.5. The observed value for the tritium isotope effect is 10 (Table II). The reason for this is not clear at present. It may indicate that the proton transfer step is not entirely rate-determining, or it may be due to quantum mechanical tunneling. Similar discrepancies between substrate deuterium and tritium kinetic isotope effects were observed with glucose oxidase (27).

Effect of $^3$H$_2$O on D-Amino Acid Oxidase Reaction

When the d-amino acid oxidase reaction was investigated in 99% $^3$H$_2$O the data of Table IV were obtained. Experiment I simply reaffirms the α-$^3$H substrate kinetic isotope effect seen above. In Experiment 2, when chloroalanine was used as substrate in $^3$H$_2$O and $^3$H$_2$O with 50% O$_2$ in the gas phase, a $k_{H^3}^{p_{H^3}}/k_{H^3}^{p_{H^3}}$ of 1.98 is observed. The same magnitude of solvent isotope effect obtains at 0% O$_2$ (Experiment 3). It is of interest to note that in Experiment 2 although the overall conversion of chloroalanine was slowed 2-fold, no differential effect was detected in formation of pyruvate and chloropyruvate. This is reminiscent of the α-$^3$H substrate effect in the last section. In Experiment 4, [α-$^3$H]-chloroalanine in $^3$H$_2$O is compared with [α-$^3$H]-chloroalanine in H$_2$O. The combined isotope effect on total keto acid production is 3.1, larger than the value of kinetic isotope effect or solvent isotope effect alone. Again there is no change in product composition. These results reinforce the conclusion reached in the last section that a common rate-determining step is involved in the formation of pyruvate and chloropyruvate. In Experiment 5, alanine was used as substrate to demonstrate that the observed solvent isotope effect is not a singular consequence of employing chloroalanine as substrate.

To ensure that the observed solvent isotope effect in $^3$H$_2$O was not a fortuitous reflection of an altered pH optimum of d-amino acid oxidase in $^3$H$_2$O, a complete pH optimum of enzyme activity in H$_2$O and in $^3$H$_2$O was recorded and is shown in Fig. 7. It is apparent that the solvent isotope effect persists over the entire pH range of enzymatic activity, $k_{H^3}^{p_{H^3}}/k_{H^3}^{p_{H^3}}$ varying from about 1.6 to 2.3. The pH optimum may be shifted somewhat to higher pH in $^3$H$_2$O.

It is incompletely understood at this time how both the solvent isotope effect and the substrate kinetic isotope effect act incrementally in the formation of a common intermediate (Experiment 4, Table IV). The observed solvent isotope effect may derive from a rapid equilibrium hydrogen transfer of solvent protons, a concerted reaction involving proton addition and abstraction, or may reflect an intrinsic lowered $V_{max}$ in $^3$H$_2$O due perhaps to a change in enzyme structure in $^3$H$_2$O.

Evidence against Equilibration of Substrate α-H with Solvent Hydrogen

To test whether the α-hydrogen of the substrate equilibrates with the solvent protons during the course of the enzymatic reaction, incubations were carried out in $^3$H$_2$O. Substrates were incubated anaerobically with the enzyme in tritiated water, then resolated, subjected to paper electrophoresis, and analyzed for tritium content. Data from incubations with proline, alanine, and chloroalanine are presented in Table V. No exchange of solvent $^3$H into any of the three amino acids was detected. Under the incubation conditions, no product formation was detected with alanine and proline. It is of interest to recall that [α-$^3$H]-serine did not lose any $^3$H into the water when it was incubated anaerobically with no net keto acid formation. About 30% of the chloroalanine was converted to pyruvate during the 10-min incubation.

![Fig. 7. $V_{max}$ of chloroalanine reaction as a function of pH or pyruvate.]
It might be argued that proline and alanine do not react reversibly with the enzyme anaerobically, that is, once the complex of imino acid and E-FADH₂ has formed it cannot revert to amino acid and E-FAD. Radhakrishnan and Meister (22) have shown this to be untenable by demonstrating such reversibility with the imino acids derived from proline and alanine. Possibly, no hydrogen incorporation occurs because E-FADH₂, imino acid decomposes much more rapidly to products than it reverts to E-FAD + amino acid. If that is not the case, then it must be concluded that E-FADH₂-imino acid does not exchange protons with the solvent. Examples of direct hydrogen transfer

**Table V**

**Evidence against equilibration of amino acid α-H with solvent protons**

Each incubation contained, in a final volume of 250 μl: 9.4 × 10³ cpm; 20 mM sodium pyrophosphate (pH 8.5); 40 μg of catalase; 150 μg of d-amino acid oxidase; 30 mM ammonium sulfate; and amino acid as indicated. Incubations were conducted anaerobically for 10 min at room temperature and terminated by immersing the mixture in dry ice-ethanol solution. The 3H₂O was removed by lyophilization. The residue was suspended in 250 μl of H₂O and lyophilized two more times. The residue was then redissolved in a small volume of water, and aliquots submitted to electrophoresis at pH 1.9 as described under “Methods.” The electropherograms were scanned for radioactivity and then stained with ninhydrin. Just prior to termination of the enzymatic incubation, a 25-μl aliquot was removed and assayed for keto acid product.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Keto acid product</th>
<th>α-H in calculated amino acid</th>
<th>α-H in amino acid for full equilibration</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Proline (4 mm)</td>
<td>0</td>
<td>None</td>
<td>2 × 10⁶</td>
</tr>
<tr>
<td>n-Alanine (5 mm)</td>
<td>0</td>
<td>None</td>
<td>5 × 10⁶</td>
</tr>
<tr>
<td>n-Chloroalanine (10 mm)</td>
<td>1.35</td>
<td>None</td>
<td>3.5 × 10⁶</td>
</tr>
</tbody>
</table>

**Table VI**

**Evidence against intermolecular transfer of amino acid α-H**

The incubation contained, in a volume of 600 μl: dl-[α-3H]proline, 8 mm (1.0 × 10³ cpm of α-H); sodium pyrophosphate (pH 8.0), 80 mM; ammonium sulfate, 40 mM; sodium pyruvate, 40 mM; and d-amino acid oxidase, 150 μg, added last. Incubation proceeded in an atmosphere of 100% N₂ for 2 hours at 25°C. The incubation was terminated anaerobically by immersion in a 95°C bath for 2 min to obtain coagulated protein which was removed by centrifugation. The supernatant was divided into two aliquots: one aliquot was evaporated to dryness, and the residue was redissolved in a small volume of water, streaked onto Whatman No. 3MM paper, and subjected to electrophoresis at pH 1.9 as described under “Methods.” The paper was subsequently scanned for radioactivity, followed by treatment with a ninhydrin dip reagent to locate amino acids. The other aliquot of reaction mixture supernatant was lyophilized and the recovered water was counted for α-H content. The lyophilized residue was subjected to electrophoresis as above, the alanine was eluted from the paper with water, an aliquot was assayed by quantitative ninhydrin and then the remainder was counted for α-H cpm.

<table>
<thead>
<tr>
<th>Initial d-proline</th>
<th>Alanine synthesized</th>
<th>α-H in water</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmoles</td>
<td>cpm</td>
<td>μmoles</td>
</tr>
<tr>
<td>2.00</td>
<td>5 × 10⁴</td>
<td>0.79</td>
</tr>
</tbody>
</table>

**Table VII**

**Products from L-Chloroalanine with L-Amino Acid Oxidase**

In a preliminary experiment with purified L-amino acid oxidase from rattlesnake venom (Crotalus adamanteus) it was observed that L-chloroalanine served as an enzymatic substrate where air was the gas phase. Chloroalanine is a relatively poor substrate for the L-amino acid oxidase, giving about 5% the Vₘαx observed with leucine, but this sufficed to determine that both pyruvate and chloropyruvate were formed enzymatically. Pyruvate comprised about 25% of the total keto acid as seen in Table VII. Thus, L-amino acid oxidase also appears to have the capability to remove Cl⁻ from chloroalanine in conversion to keto acid product. A thorough study is required to define the characteristics of this catalytic activity of L-amino acid oxidase.

**Table VIII**

**Products from L-Chloroalanine with L-amino acid oxidase**

Each incubation contained, in 2.5 ml: 50 mM sodium pyrophosphate (pH 8.0); 8 mM L-isomer of chloroalanine; 110 μg of catalase; and 440 μg of L-amino acid oxidase to start the reaction. Aliquots of 200 μl were removed at intervals and assayed both for pyruvate and for total keto acid content. Product compositions were constant at intervals of 10, 15, and 15 min. Incubations were in 20% oxygen.
DISCUSSION

\[ \text{CICH}_2\text{-CH-COO}^- + E\cdot\text{FAD} \rightarrow \text{CICH}_2\text{-CH-COO}^- + H_2\text{O} \]

D-Amino acid oxidase catalyzes the oxidation of \( \alpha \)-chloroalanine to chloropyruvate as well as its nonoxidative conversion to pyruvate. Under anaerobic conditions chloroalanine is converted exclusively to pyruvate, whereas in an atmosphere of 100% \( O_2 \) chloropyruvate is the predominant product. At intermediate oxygen concentrations mixtures of the two products are formed. The overall rate of reaction, i.e. the rate of formation of pyruvate plus chloropyruvate, is independent of \( O_2 \) concentration.

When the \( \alpha \)-hydrogen of the substrate is substituted by deuterium or when the reaction is carried out in deuterium oxide, the rate of total product formation (pyruvate + chloropyruvate) is reduced but the relative distribution of products is not changed. These results are consistent with the rate-determining formation of a common precursor of both chloropyruvate and pyruvate. The partitioning of the precursor between the two products is determined by the \( O_2 \) concentration. The occurrence of \( \alpha\text{-H} \) and \( \alpha\text{-D} \) isotopic effects indicate that the dissociation of the \( \alpha\text{-C-H} \) bond of chloroalanine is rate-limiting, or partially rate-limiting. Furthermore, since the formation of pyruvate from chloroalanine most likely involves removal of the \( \alpha \)-hydrogen as

\[ \text{CICH}_2\text{-CH-COO}^- + \text{FAD} \rightarrow \text{CICH}_2\text{-CH-COO}^- + \text{FAD} \]

In view of the effect of \( O_2 \) on product composition, the exist-
ence of a complex consisting of the substrate-carbanion, or an intermediate in equilibrium with the carbanion), FAD, apoenzyme, and O₂ must be postulated. Such a complex is consistent with the mechanism proposed for d-amino acid oxidase by Massey and Gibson (1) based on kinetic analysis. Both these workers, as well as Koster and Veeger (3), have maintained that, despite parallel line kinetics, ternary complexes of E-FAD, O₂, and imino acid are kinetically significant, and that dissociation of imino acid product is a relatively slow step. In view of our finding that the substrate deuterium kinetic isotope effects are low enough perhaps to be only partially rate-determining, it may well be that a slow dissociation of the imino acid products is also partially rate-determining with the substrates we have studied. A tentative reaction sequence, incorporating such observations, and involving an intermediate carbanion, is shown in Scheme 2. This scheme also accounts for the observation (e.g. Fig. 4) that under anaerobic conditions the production of pyruvate gradually decreases and ceases completely. It is proposed that the imino acid slowly dissociates from Complex III. This gives rise to E-FADH₂, catalytically inactive with chloroalanine. Massey et al. (33) have demonstrated that additions at N₁ of imino acid, which rapidly hydrolyzes, thus prevent regeneration of III. For such a process to be operative, dissociation of the imino acid must be slow relative to the sequence of reactions leading to pyruvate. The above proposal is consistent with the observation that admission of O₂ to the anaerobic incubation after pyruvate production has ceased results in regeneration of active enzyme.

Scheme 2 represents the simplest mechanism which can reasonably account for our experimental data. It is not necessarily our conclusion that electron transfer occurs between the free substrate carbanion and FAD, although such electron transfer has been proposed recently for flavin model systems (31). A reasonable mechanism has been proposed which involves formation of an adduct between amino acid and FAD, followed by simultaneous a-proton abstraction and electron transfer from the substrate to the flavin. Scheme 3 shows a partial reaction sequence, adapted from a mechanism proposed by Brown and Hamilton (5), for the formation of chloropyruvate (solid arrows) and pyruvate (dashed arrows). Other possible intermediates, suggested by the work of Hemmerich (32) are substrate-FAD adducts in which the substrate adds to N³ of the flavin as indicated in Fig. 8. Such a structure could break down to chloropyruvate (solid arrows) or pyruvate (dashed arrows). The electron deficient carbons, Cₓ and Cᵧ, facilitate Michael type additions at N² Massey et al. (33) have demonstrated that nucleophilic sulfite adds to the N³ position of the flavin coenzyme in d- and l-amino acid oxidases. Further experimental evidence will be needed to decide which, if any of these intermediates, is involved.

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