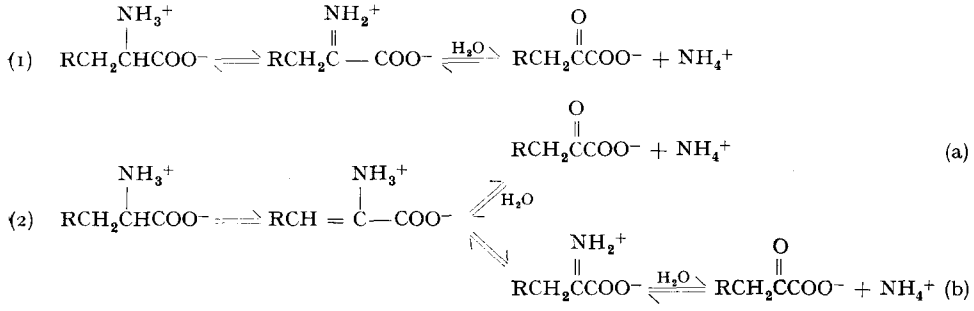
Imino acid as the intermediate in the L-amino acid oxidase reaction

It has been generally assumed that the primary product of the dehydrogenation of amino acids catalyzed both by pyridine nucleotide and by flavin nucleotide enzymes is an unstable imino acid which hydrolyzes non-enzymically to the corresponding keto acid. This idea was based on reasonable chemical considerations[1]. However, there is no direct experimental evidence for the occurrence of the imino acid as the primary reaction product. Two alternative mechanisms are:



The primary product of mechanism 2 is a dehydro amino acid. The plausibility of the occurrence of such an intermediate is shown by the behavior of peptides of dehydro amino acids in enzymic hydrolysis[2].

A choice between the above two mechanisms may be made from enzymic amino acid oxidations carried out in a medium of D_2O. When the proper control for enolization of the product is made, mechanism (1) should yield a keto acid containing no deuterium, while mechanism (2) should result in the formation of a keto acid containing at least one atom of deuterium on the β carbon atom.

Enzyme. Ophio-L-amino acid oxidase of rattlesnake venom* was isolated by a method similar to that of SINGER AND KEARNEY[3]. Approximately 1 g of venom was dissolved in water and fractionated with ammonium sulfate at 25°. The precipitate from the 62 to 90% saturated ammonium sulfate fraction was dissolved in tris(hydroxymethyl)aminomethane acetate, pH 7.2, 0.25 mmoles of L-leucine added, and the solution heated to 73° for five minutes. After cooling, the precipitate was spun off and discarded. The supernatant solution was again fractionated with ammonium sulfate, the fraction between 65 and 70% saturation being used in the experiment. From the measurement of the reaction rate (see below) and optical density readings at 280 mμ, it was shown that the enzyme in this fraction was purified approximately 25 fold over the starting material.

Enzymic activity. The activity of the oxidase was determined using a spectrophotometric assay system with L-leucine as substrate. It was found that in neutral solutions and in the region 210 to 230 mμ L-leucine has a rather small extinction coefficient compared to α-keto *iso*caproate, the product of the reaction. Thus, the activity of the enzyme was determined from the change of optical density at 220 mμ of one ml of solution 0.005 M in L-leucine, 0.01 M in tris chloride at pH 7.8 and containing approximately 15 units of crystalline catalase**. The 80 to 100% transmission expanded scale of a Brown recorder coupled to a Beckman DUR spectrophotometer allows a rather accurate measurement of the initial velocity of the reaction before the dissolved oxygen in the solution is depleted.

Isotopic experiment. Approximately 250 mg of L-leucine were dissolved in 10 ml of 95% D_2O, which was 0.025 M in tris chloride at pH 7.8 and which contained 400 units of crystalline catalase and approximately 3 mg of L-amino acid oxidase. Dry oxygen was bubbled through the solution and the reaction was allowed to proceed at room temperature for 5 hours. At the same time, approximately 20 mg of α-keto *iso*caproate were dissolved in 1 ml D_2O at the same pH and buffer concentration with no enzyme present. This non-enzymic control was allowed to stand for 5½ hours.

Isolation of products. At the end of 5 hours the enzymic reaction mixture was poured on a column of Dowex-50 (acid form, washed). Water was then passed through the column[4] at the rate of 2–3 ml per 5 minutes. Within 40 minutes all the α-keto acid had been washed from the column. A 1% solution of 2,4-dinitrophenylhydrazine in 2 N HCl was added to the keto acid solution and the hydrazone derivative immediately precipitated. The hydrazone was filtered, air-dried, washed in water and finally dried over P_2O_5. The α-keto *iso*caproate in the non-enzymic

* Purchased from Ross Allen's Reptile Institute, Silver Springs, Florida.

** Obtained from the Worthington Biochemical Corp., Freehold, New Jersey.

control was isolated directly as the hydrazone derivative. In an experiment similar to that described in the last section, the unoxidized L-leucine remaining on the column after washing with water was eluted with 2 *N* HCl. The eluate was evaporated to dryness several times in a rotating evaporator. The residue was dissolved in water, cleared by centrifugation and neutralized with 1.6*N* NaOH. Five volumes of alcohol were added and the solution cooled and the crystalline L-leucine centrifuged off. The leucine was then recrystallized in the same manner and finally taken to dryness over P_2O_5.

The isolated compounds were then analyzed for deuterium content*. The results are shown in Table I.

TABLE I

Compound recovered	*moles deuterium/mole*
L-leucine	~0
Phenyl hydrazone (enzymic reaction)	0.12
Phenyl hydrazone (non-enzymic reaction)	0.15

It is unlikely that any significant exchange occurred on the column since the α-keto *iso*caproic acid is not bound to the resin and the medium in which the acid is eluted from the column is at least 50% D_2O.

Thus it is quite apparent that the deuterium which is incorporated into the α-keto *iso*caproate is a result of enolization only and that no other deuterium from the medium is incorporated. Furthermore no significant reversal occurred under the reaction conditions since no excess deuterium is incorporated into the L-leucine. The oxidation of amino acids by L-amino acid oxidase must therefore proceed through the imino form of the amino acid according to mechanism (1) and an α–β unsaturated intermediate as in mechanism (2) is not formed.

Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Mo. (U.S.A.) CARL FRIEDEN
SIDNEY F. VELICK

[1] H. v. EULER, E. ADLER, G. GUNTHER AND N. B. DAS, *Z. physiol. Chem.*, 254 (1938) 61.
[2] J. P. GREENSTEIN, *Advances in Enzymol.*, 8 (1948) 117.
[3] T. P. SINGER AND E. B. KEARNEY, *Arch. Biochem.*, 29 (1950) 190.
[4] A. MEISTER, *Biochem. Preparations*, 3 (1953) 66.

Received November 17th, 1956

* DR. GEORGE R. DRYSDALE kindly performed these analyses.

Interference by reduced pyridine nucleotides in the diazotization of nitrite

Nitrate and nitrite reductases are usually assayed in plant extracts by determining the formation or removal of NO_2^-, using either di- or triphosphopyridine nucleotide (DPNH or TPNH) as an electron donor[1,2,3,4]. Sulphanilamide is added to form a diazo compound with NO_2^-, which is then coupled to N-(1-naphthyl)-ethyldiamine hydrochloride to form the red azodye[5]. It has been observed that a small non-enzymic disappearance of nitrite occurs when TPNH and nitrite are allowed to react for a few minutes with the acid sulphanilamide reagent prior to coupling it to the naphthyl reagent[6]. This has been attributed to the deamination of the amino group of the adenine in TPNH.

During the course of our enzymic studies on nitrate assimilation in plants, we have confirmed that TPNH or DPNH interfere with the nitrite test. When determining the appearance of NO_2^- from NO_3^- in the nitrate reductase assay the effect of the TPNH or DPNH is negligible because NO_2^- is being formed. There is, however, considerable interference in the nitrite reductase method, because NO_2^- is being removed and the DPNH required for maximal activity of the enzyme results in more than 75% non-enzymic disappearance of NO_2^-, as shown in Table I.

TABLE I

ASSAY OF NITRITE REDUCTASE FROM *Neurospora crassa*

(mμmoles NO_2 disappearing/10 min incubation period)

Experiment	*1*	*2*	*3*
Complete reaction mixture	15	14	16
Reaction mixture less enzyme	9.4	11.1	11.1

Complete reaction mixture: 0.15 ml 0.1*M* pyrophosphate (pH 7.5); 0.2 ml 10^{-4}*M* $NaNO_2$; 0.05 ml boiled pig heart; 0.1 ml 10^{-3}*M* DPNH and 0.1 ml enzyme (1.56 mg protein/ml). After 10 min incubation, 0.5 ml 1% w/v sulphanilamide in *N* HCl and 1 ml 0.01% w/v N-(1-naphthyl)-ethylenediamine hydrochloride were added and the volume made to 5 ml with distilled water. The solutions were assayed in a Spekker absorptiometer in 0.5 cm cells, with green filters.