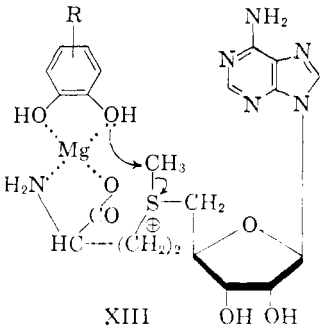
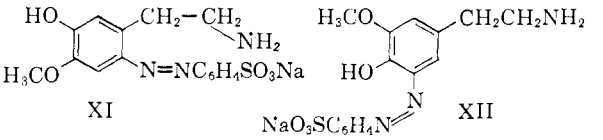


XIII

cholate anion[16] IIIc starts competing successfully with the uncharged species IIIa. To what extent binding by additional functional groups enters into the (magnesium-requiring) nucleophilic displacement on S-adenosylmethionine (hypothetical formulation XIII[17]) is not known. The steric requirements with respect to the sulfonium center are very selective and are reported to be identical for catechol O-methyl transferase, guanidinoacetate methylpherase and the S-adenosylmethionine cleaving system from yeast.[18]

The mechanism of the enzymatic *p*-O-methylation of 3,4-dihydroxyphenylmethylcarbinol (Va) or of dopamine (VIa) *in vitro* cannot readily be rationalized. The physiological implications are of great interest, should this process also occur *in vivo*.

The azo coupling products, *e.g.*, of the O-methylated dopamines VIb and VIc may be formulated as



XI XII

XI and XII. The different points of attachment of the azo part and possible (non-bonded) interactions with the side chain in XI or the phenolic hydroxyl in XII facilitate chromatographic separation and may cause the striking differences in color. "Paranephrine," *i.e.*, *p*-O-methylepinephrine,[19] with regard to pressor effects, is no more active than metanephrine.[20] Investigations now in progress[21] are aimed at establishing the ratio of formation of (nor)-paranephrine *in vitro* and *in vivo* as well as any possible indications for its being a better substrate than (nor)metanephrine for the demethylase that reconverts such ethers to (nor)epinephrine.[22] Previous data indicate that *p*-O-alkyl ethers are cleaved more rapidly than their *m*-O-methyl analogs.[23]

Acknowledgment.—We are greatly indebted to Dr. Sydney Archer, Sterling-Winthrop Research Institute, for his interest and donation and preparation of compounds.

(16) *Cf.* J. W. Churchill, M. Lapkin, F. Martinez and J. A. Zaslowsky, THIS JOURNAL, **80**, 1944 (1958).

(17) *Cf.* J. C. Bailar, Jr., "The Chemistry of the Coördination Compounds," Reinhold Publ. Corp., New York, N. Y., 1956, p. 698.

(18) G. de la Haba, G. A. Jamieson, S. H. Mudd and H. H. Richards, THIS JOURNAL, **81**, 3975 (1959).

(19) *Cf.*, F. Külz and W. Hornung, German Patent 682,394 (1939); *C. A.*, **36**, 3011 (1942).

(20) We are greatly obliged for this information to Drs. S. Archer and A. M. Lands, Sterling-Winthrop Research Institute.

(21) *Cf.*, J. Daly, J. Axelrod and B. Witkop, "Dynamic Aspects of Enzymatic O-Methylation and -Demethylation of Catechols *in Vitro* and *in Vivo*," *J. Biol. Chem.*, in preparation.

(22) J. Axelrod and S. Szara, *Biochim. Biophys. Acta*, **30**, 188 (1958).

(23) J. Axelrod, *Biochem. J.*, **63**, 634 (1956).

BETHESDA 14, MD.

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE, U. S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE]

Chemical and Enzymatic Studies of the Labile Metabolite 4(5H)-Imidazoline-5-acetic Acid[1]

BY HERMANN KNY[2] AND BERNHARD WITKOP

RECEIVED MAY 15, 1959

Carefully controlled intramolecular cyclization of α-ethyl formimino-L-aspartate (II) yielded 4(5H)-imidazolone-5-acetic acid (III) which had a half-life time of close to one hour at *p*H 8. Its *enzymatic degradation* by imidazoleacetic acid oxidase from *Pseudomonas* was followed *in situ* in a self-recording spectrophotometer by the disappearance of λ_{max} 259 mμ; it was found to be significantly faster than the *spontaneous hydrolysis* and produced more formiminoaspartic acid (VII) than the spontaneous decomposition which yielded more (formyl)isoasparagine, pointing to two different sites for cleavage on the imidazolone ring. Translactamization of III to 5,6-dihydro-4-pyrimidone-6-carboxylic acid (VIII) could not be ruled out. The highly unstable dihydropyrimidone VIII was the intermediate in the partial hydrogenation of the pyrimidone IX with a rhodium catalyst as evidenced by the appearance of formiminoaspartic acid (VII) besides asparagine which probably arose from the accompanying tetrahydropyrimidone XII *via* methyleneasparagine (XI). The internal cyclization of formylasparagine (XV) or -isoasparagine (IV) with acetic anhydride did not yield the dihydropyrimidone VIII but 3-formaminosuccinimide (XVII).

Imidazoleacetic acid is degraded by a strain of *Pseudomonas fluorescens*, adapted to imidazoleacetic acid, to formiminoaspartic (VII)[3] and formylaspartic acid (VI).[4] It is known that O^{18} is directly incorporated into imidazoleacetic acid[5] by a "mixed function oxidase."[6] The presumed intermediate

(1) Labile Metabolites. VIII. Preceding paper, *cf.* THIS JOURNAL, **81**, 1768 (1959).

(2) Visiting Scientist of the USPHS from the University of Basle, Switzerland.

(3) O. Hayaishi, H. Tabor and T. Hayaishi, THIS JOURNAL, **76**, 5570 (1954).

(4) O. Hayaishi, H. Tabor and T. Hayaishi, *J. Biol. Chem.*, **227**, 161 (1957).

(5) O. Hayaishi, "Proc. Internat. Symposium on Enzyme Chemistry," Tokyo and Kyoto, 1957, p. 207.

(6) *Cf.* H. S. Mason, "Advances in Enzymology," Interscience Publishers, Inc., New York, N. Y., Vol. 19, 1957, p. 179.

4(5H)-imidazolone-5-acetic acid (III) has not been detected, nor was it known whether the further degradation to formiminoaspartic acid (VII) involved enzymatic or spontaneous hydrolysis. The studies reported here resolve these questions.

The great lability of 4(5H)-imidazolones[7] lacking a stabilizing substituent in the 2-position[8] called for a mode of synthesis under mildest conditions, *e.g.*, by the intramolecular displacement reaction on a suitable ester in analogy to a previous approach.[7] The requisite α-ethyl formimino-L-aspartate was prepared by condensing formamidine with α-ethyl L-aspartate[9] in formamide.[10] The conditions for this condensation had to be worked out very carefully. The ester hydrochloride II was obtained by gradient elution from a Dowex 50-H^+ column with hydrochloric acid. It was not possible to obtain this ester hydrochloride crystalline either by lyophilization of the solution or by precipitation as a salt, *e.g.*, as the reineckate. Its presence was proved by liberation of the free ester of II which, as expected, cyclized immediately to the imidazolone III recognizable from its absorption peak at 260 mμ.

Figure 1 shows that the rate of formation of the imidazolone III is a function of *p*H. Within the range of *p*H 9.6–10.2 the reaction obeys first-order kinetics. At lower *p*H the formation of III is too slow and the curves are deformed by competing side reactions; at higher *p*H decomposition and formation of III become competitive. The rate constants of Fig. 1 are initial rates only, representing the average of 4–6 measurements for each value. These figures served as a guide for the optimal conditions for the formation of the imidazolone III, namely, short exposure (30 sec.) of the ester hydrochloride to *p*H 10.5 and rapid adjustment to *p*H 7 or any other desired *p*H. This procedure minimized side reactions and decomposition.

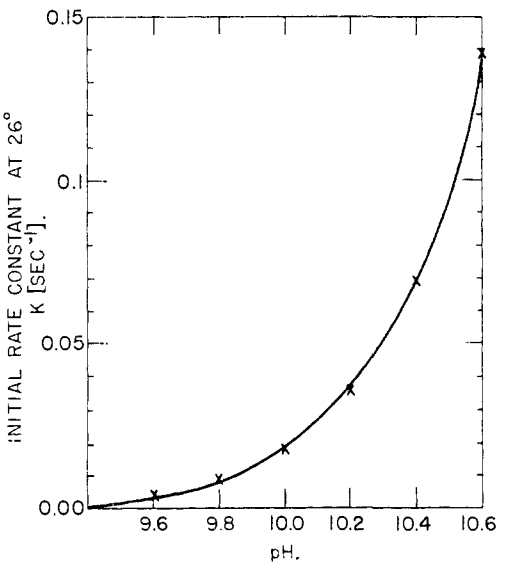


Fig. 1.—Rate of formation of 4(5H)-imidazolone-5-acetic acid on liberation of the ester from the hydrochloride of α-methyl formiminoaspartate in aqueous 1/10 *N* buffer solutions (glycine–sodium hydroxide) of varying *p*H.

We studied next the rate of disappearance of the imidazolone III with and without imidazoleacetic oxidase as a function of *p*H. The results, summarized in Fig. 2, reveal a striking and unexpected difference. The non-enzymatic disappearance is slowest at *p*H 7–8 and becomes rapid >*p*H 9.5, whereas the enzymatic degradation is fastest at *p*H 7 and slow at *p*H 10. The presence of the enzyme seems to have a protective influence on the substrate at *p*H >9. One might assume that chelation could have a stabilizing effect but, unlike the enzymatic hydrolysis of 4(5H)-imidazolone,[7] that of III showed no requirements for manganese or iron salts and no inhibition by Versene or 8-hydroxyquinoline-5-sulfuric acid (Table I). Care had to be taken in these inhibition studies to work in an inert system, such as phosphate buffer, since Tris buffer was found to have a slight but noticeable inhibitory effect at $^1/_{10}$–$^1/_{100}$ *M* concentration. Other derivatives of ethanolamine or ethylenediamine had no inhibitory effect. Unfortunately, the spectrophotometric technique allowed rate studies only down to *p*H 7 where λ_{max} 259 mμ of the imidazolone III starts disappearing.

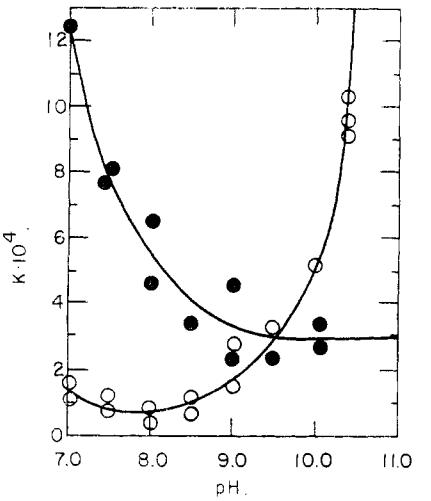


Fig. 2.—Dependence of the enzymatic (—●—●—) and non-enzymatic (—O—O—) disappearance of the 259 μ peak of 4(5H)-imidazolone-5-acetic acid on the *p*H of the solution as measured in buffer solutions (phosphate below *p*H 8.5, glycine–sodium hydroxide above *p*H 8.5). Measurements below *p*H 7 are impossible because of disappearance of the typical imidazolone band.

The enzymatic rate at *p*H 7 (Fig. 2) may not be the optimal rate. In any case, it is significantly lower than at *p*H 8.9 at which imidazoleacetic acid oxidase is normally employed or the optimal *p*H 9.0 for formiminoaspartic acid hydrolase.[4] Although there is no proof so far for the existence of a separate and specific "*imidazoloneacetic acid hydrolase*," there is no doubt that the crude enzyme extract from *Pseudomonas* markedly accelerates the disappearance of the imidazolone, as is also shown in Fig. 3.

(7) K. Freter, J. Rabinowitz and B. Witkop, *Ann.*, **607**, 174 (1957).
(8) A. Kjaer, *Acta Chem. Scand.*, **7**, 1017 (1953).
(9) W. J. Le Quesne and G. T. Young, *J. Chem. Soc.*, **24** (1952).
(10) *Cf.* H. Tabor and J. C. Rabinowitz, *Biochem. Preparations*, **5**, 100 (1957).

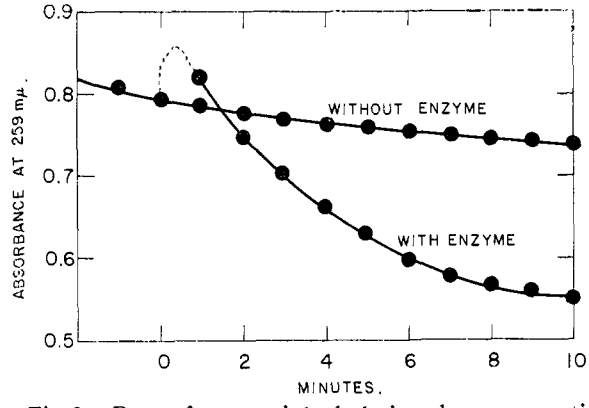
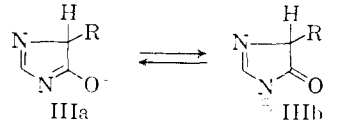
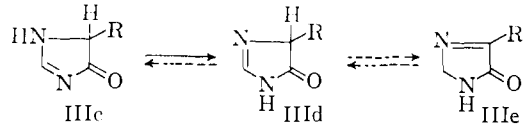


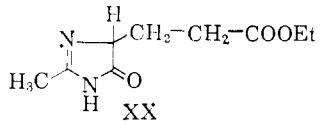
Fig. 3.—Rates of enzymatic hydrolysis and non-enzymatic decomposition of 4(5H)-imidazolone-5-acetic acid at *p*H 7 immediately (~2 min.) after its formation as measured by the disappearance of the ultraviolet absorption peak at 259 μ in a self-registering Cary spectrophotometer.

The chromophore responsible for the 259 mμ peak is apparently the enolate IIIa ⟷ IIIb. In neutral solution imidazolones show only a shoulder at this





point[7] which however for the related ethyl 2-methyl-4(5H)-imidazolone-5(4)-propionate (XX)[11] is miss-



ing. The enol–ketone equilibrium of 4-imidazolones undoubtedly is dependent on the degree and nature of ring substitution. Additionally, the tautomers IIIc, IIId, IIIe may be considered in acid solution. IIIc violates the principle of double bond shift observed in the comparable acylation of an imine to an acylvinyl amine; IIIe would be optically inactive and not easily lead to L-formiminoaspartic acid. Previous spectrophotometric studies on 4-imidazolones with additional substituents in positions 1 and 5[12,13] permit no clear decision for unsubstituted imidazolones.

The marked shift of the infrared peak of the amide carbonyl from 5.79 μ ($CHCl_3$) → 5.63 μ (Nujol) on salt formation of 2-methyl-4-imidazolone may point to uncoupling of conjugation (XXI → XXII) although this is by no means certain. It is hoped that current nuclear magnetic reso-

(11) This compound, m.p. 237–239°, was synthesized by Dr. T. W. Beiler by condensation of diethyl glutamate with acetiminoethyl ether.

(12) J. T. Edward and E. F. Marthew, *Chemistry and Industry*, 193 (1954).

(13) H. C. Carrington, C. H. Vasey and W. S. Waring, *ibid.*, 377 (1954).

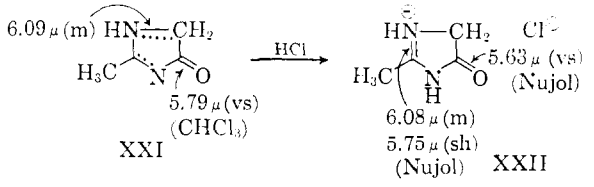
TABLE I

INHIBITION OF THE ENZYMATIC HYDROLYSIS OF IMIDAZOLONEACETIC ACID (III)

Inhibitor	Concn., *M*	% Inhibition[a]
Versene	10^{-3}	0
8-Hydroxyquinoline-5-sulfuric acid	10^{-4}	0
Tris buffer (tris-(hydroxymethyl)-aminomethane)	10^{-1}	100
	10^{-2}	~50
1,2-*cis*-Dimethylaminocyclohexanol	10^{-3}	0
Streptamine	4.10^{-3}	0
N-Dimethylaminoethylpiperidine	10^{-3}	0
p-Chloromercuribenzoic acid (PCMB)	10^{-4}	100
p-Chloromercuriphenylene sulfuric acid (CMPS)	10^{-4}	100
Enzyme heated to 98° for 5 min.		(100)

[a] The mixture of 0.15 ml. of 0.5 *M* phosphate buffer, *p*H 7.0, 50 λ of enzyme and 0.3 ml. of inhibitor solution was brought to a total volume of 1.5 ml. by the addition of water in a quartz cell. In a second cell a similar solution without the inhibitor was prepared. Both cells were stored at 25° for 10 min. Both solutions then were mixed simultaneously with 1.5 ml. of a solution of freshly-formed imidazolone-acetic acid and the decrease of absorption at 259 mμ was followed at intervals of 1 min.

nance studies will shed light on this tautomerism which might play a role in the dualism of the enzymatic and non-enzymatic cleavage reactions.



A plot of absorbance at 259 mμ *versus* *p*H (Fig. 4) might be expected to yield a curve the slope of which would reveal the halfway point where equal

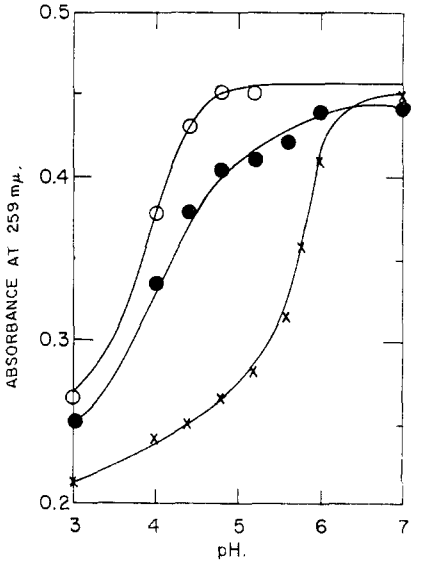


Fig. 4.—Dependence of absorbance at 259 μ on variations in *p*H in acetate and phosphate buffer solutions after 5 (—●—●—) and 30 (—x—x—) minutes after the formation of 4(5H)-imidazolone-5-acetic acid (at *p*H 10.5 for 30 sec. and rapid adjustment to the desired *p*H). The curve for zero time (—O—O—) was extrapolated from five different isochronic curves.

amounts of enolate (IIIa ⇄ IIIb) and ketone (IIIc, IIId, IIIe) are present in equilibrium, *i.e.*, the pK' of III. This was not possible, because the slope of such curves varied with time. An extrapolation from several curves at different times furnished a zero time curve (Fig. 4) which approximates a pK' around 4 but cannot claim sufficient accuracy.[14] Addition of base to the various mixtures represented by points on these curves gives back 80–90% of the original extinction at 259 mμ. Much of the extinction at lower *p*H may be due to "background" and decomposition. A transient peak of low extinction at 300 mμ is observed immediately after bringing basic solutions of III to acid *p*H.

Unlike formiminoglycine, formiminoaspartic acid could not be assayed colorimetrically in solution using the ferricyanide–nitroprusside test.[15] A semi-quantitative assay on paper (Fig. 5) showed clearly that formiminoaspartic acid (VII) was one

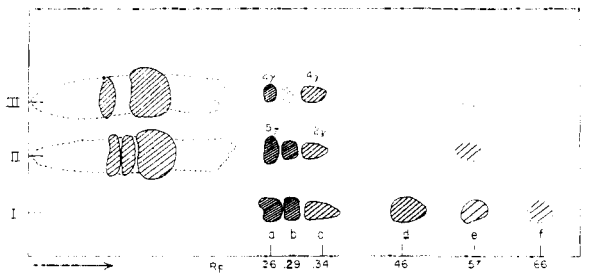
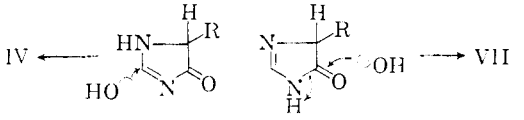


Fig. 5.—Semi-quantitative chromatograms in butanone–propionic acid–water (15:5:6) of (I) standard samples (10 γ): a (aspartic acid), b (isoasparagine), c (formiminoaspartic acid), d (formylasparagine), e (formylisoasparagine), f (formylaspartic acid), (II) of the non-enzymatic decomposition and (III) of the enzymatic decomposition of 4(5H)-imidazolone-5-acetic acid. The yield of FAA (c) in the latter case is about twice as much as in II. The spots were developed by ninhydrin spraying after treatment with gaseous NH_3 followed by HCl.

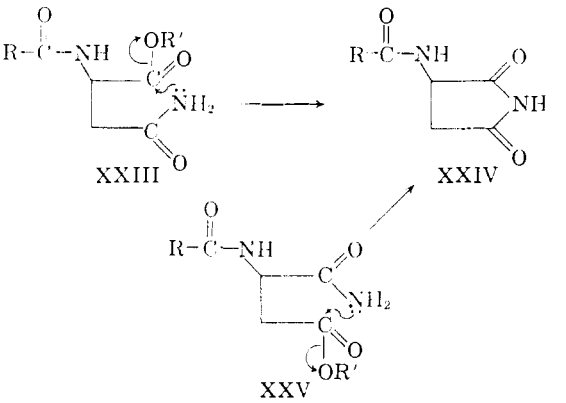
of the major products in the enzymatic degradation and that more of it was formed than in the spontaneous degradation, which furnished formylisoasparagine (IV) and isoasparagine (V). The same dualism of cleavage has been noted in the enzymatic and non-enzymatic degradation of urocanic acid.[16] It



means that the attack of hydroxyl ion or another nucleophilic species occurs at positions 2 and 4 at two centers which differ (i) sterically, (ii) with regard to relative electronegativity and (iii) by possibly being part of two tautomeric systems. To what extent anaerobic and aerobic conditions influence this dualism of cleavage (*cf.* imidazolonepropionic acid, ref. 14) has not been determined. Hydantoin-5-acetic acid[17] was not present among the products of hydrolysis or decomposition. Opening to VII would connote the acyl–amidine bond as an unstable ("energy-rich") bond, which could conceivably favor translactamization reactions to the dihydropyrimidone VIII, a type of reaction with many precedents, *e.g.*, in the oxindole series.[18] An obvious question is then whether the stability of the six-membered cyclic acyl-amidine would be greater than that of the imidazolone and whether the dualism of ring cleavage would still obtain.

The synthesis of the dihydropyrimidone VIII was therefore undertaken. It was expected that dehydration with acetic anhydride of formylasparagine should yield the desired dihydropyrimidone (VIII), in analogy to oxidative cyclization of methyleneasparagine (XI) to the aromatic pyrimidonecarboxylic acid IX.[19] However, the reaction took the same course as the thermal condensation of N-benzoylasparagine to N-benzoylaminosuccinimide.[19,20] The same formamidosuccinimide XVII also was obtained from the newly prepared formylisoasparagine with acetic anhydride. In both cases a labile compound was formed in 30% yield from XV and in 70% yield from IV which easily was transformed on standing or on rechromatography to formamidosuccinimide (XVII).

The formation of the imide from XV and IV is analogous to the intramolecular cyclization of N-carbobenzyloxy-L-asparagine α-methyl ester



(XXIII, R = $C_6H_5CH_2O$, R′ = CH_3) and the isomeric XXV (R = $C_6H_5CH_2O$, R′ = CH_3) to the imide XXIV (R = $C_6H_5CH_2O$).[21] Whereas the latter reaction is base-catalyzed and leads to departure of methoxide, the acetic anhydride reaction probably involves activation of the carboxyl group by mixed anhydride formation (XXIII, XXV: R′ = CH_3CO) and intramolecular elimination of acetate ion.

Spectrophotometric analysis of the products from the reaction of formiminoaspartic acid with diazomethane and subsequent action of base shows the presence of two products with λ_{max} 257 and λ_{max} 221

(14) 4(5H)-Imidazolone-5(4)-propionic acid recently synthesized by enzymatic methods gave a pK' ~4.4 by plotting ratio λ_{max} 260/234 *versus* *p*H (private communication from Dr. D. D. Brown, NIMH).

(15) J. C. Rabinowitz and W. E. Pricer, Jr., *J. Biol. Chem.*, **222**, 537 (1956).

(16) H. R. B. Revel and B. Magasanik, *ibid.*, **233**, 930 (1958); R. H. Feinberg and D. M. Greenberg, *ibid.*, **234**, 2670 (1959).

(17) *Cf.* D. D. Brown and M. W. Kies, THIS JOURNAL, **80**, 6147 (1958).

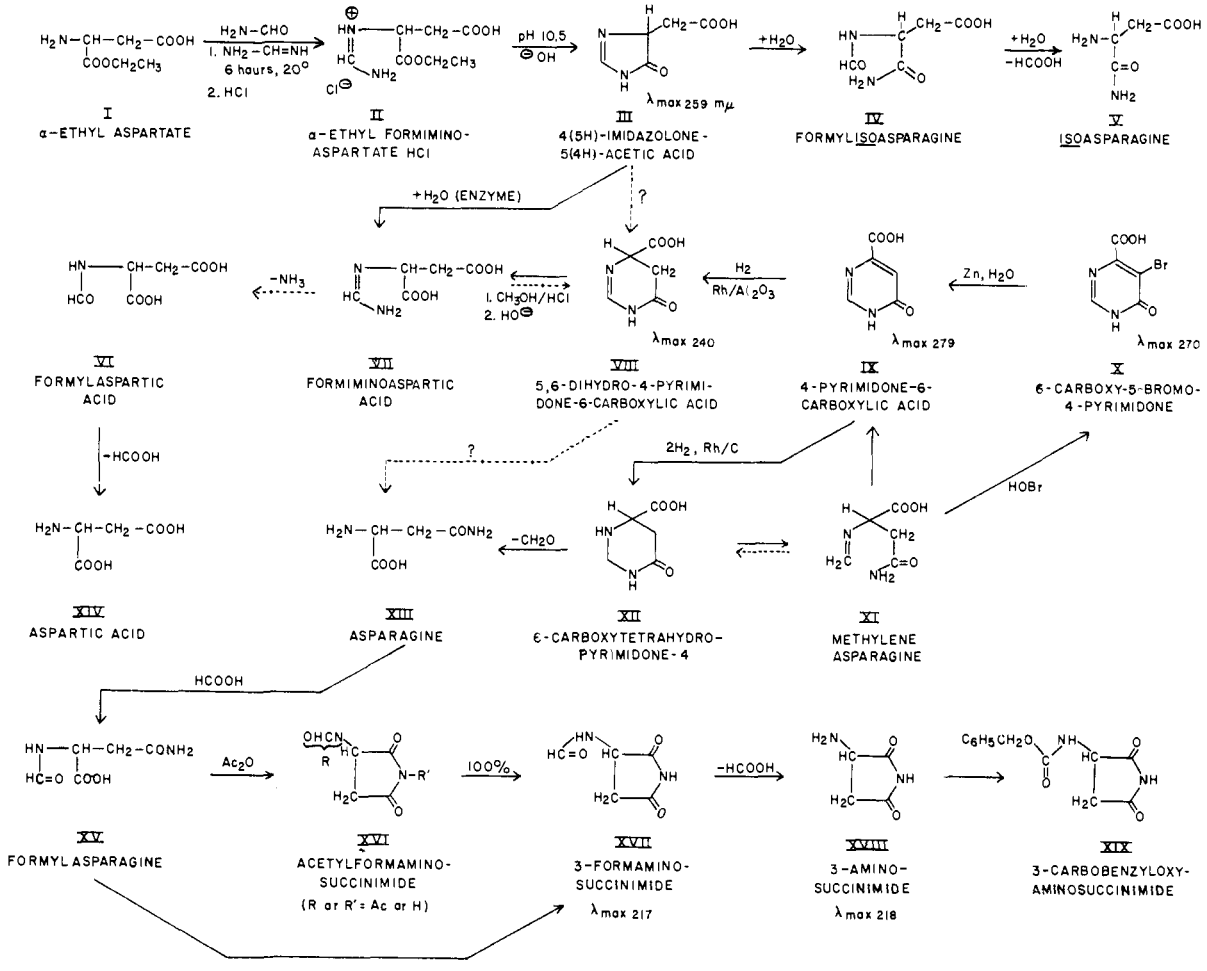
(18) P. L. Julian, *ibid.*, **75**, 5305 (1953).

(19) E. Cherbuliez and K. N. Stavritch, *Helv. Chim. Acta*, **5**, 267 (1922).

(20) *Cf.* A. R. Battersby and J. C. Robinson, *J. Chem. Soc.*, 259 (1955).

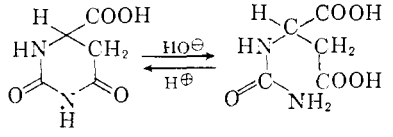
(21) E. Sondheimer and R. W. Holley, THIS JOURNAL, **76**, 2467 (1954); and ref. 20.

CHART I. FORMATION OF 4(5H)-IMIDAZOLONE-5(4)-ACETIC ACID AND ITS ENZYMATIC AND NONENZYMATIC TRANSFORMATIONS



mμ. One is the imidazolone, the other might be the dihydropyrimidone. A preparative approach *via* formimination of β-methyl aspartate was not successful.

A synthesis of the desired dihydropyrimidone started with the pyrimidone IX or its 5-bromo derivative X, both available from methylene asparagine by the action of permanganate or hydrobromous acid.[19] Partial hydrogenation of IX with a rhodium on alumina catalyst,[22,23] or dehalogenation of X (Pd) and hydrogenation (Rh, Al_2O_3) under a variety of conditions carried out on a normal or micro (Warburg) scale, invariably led to mixtures whose chromatographic analysis revealed the presence of the three major components: formiminoaspartic acid (VII), which can only come from the highly unstable dihydropyrimidone VIII; methyleneasparagine (XI), which is the chain tautomer of the less stable tetrahydropyrimidone XII; and asparagine whose origin could be from either VIII or XII *via* XI. Whether asparagine comes from one or both of these sources, a decision was not possible, since the hydrogenation of the pyrimidone IX could not be arrested at the dihydro stage.



Dihydroörotic acid — Ureidosuccinic acid

The ring-chain equilibrium of VIII (2-deoxyorotic acid) is completely on the side of the open formiminoaspartic acid (VII) in contrast to the easy acid-catalyzed reclosure of ureidosuccinic acid or β-ureidopropionic acid to dihydroörotic acid[24] or dihydrouracil.[25] Substituents alter the ease of alkaline cleavage of dihydropyrimidines. Dihydrouracil and uridine show higher rates of cleavage than dihydrothym(id)ine.[22]

A careful study of the degradation products of imidazoloneacetic acid (III) in buffer solutions of varying *p*H gave no indication for the presence of (formyl)-asparagine. This finding, however, does not rule out the dihydropyrimidone VIII as a pos-

(22) M. Green and S. S. Cohen, *J. Biol. Chem.*, **225**, 397 (1937).

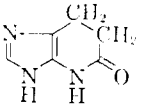
(23) W. E. Cohn and D. G. Doherty, THIS JOURNAL, **78**, 2863 (1956); *cf.* A. Bendich, "Chemistry and Biology of Purines," Ciba Foundation Symposium, 1957, Little, Brown & Co., Boston, Mass., 1957, p. 308.

(24) I. Lieberman and A. Kornberg, *J. Biol. Chem.*, **207**, 911 (1954).

(25) L. Campbell, *ibid.*, **233**, 1236 (1958).

sible or alternate intermediate on the way to formiminoaspartic acid.

The preparation of the homologous imidazolonepropionic acid[26] by formimination of α-methyl glutamate and base-catalyzed cyclization under a variety of conditions did not succeed. An alternate route *via* reduction and diazotization of 4(5H)-nitroimidazole-5(4)-propionic acid met with what is believed to be the complication of lactam (XXVI) formation.[27]



XXVI

Experimental[28]

Spectrophotometric studies were made on a Cary Model 14 recording spectrophotometer. Paper chromatographic assays were run on Whatman paper No. 1 and 7 using the following solvent systems: Solvent system I, 1-butanol–acetic acid–water 70:15:15 (vol.); solvent system II, butanone–propionic acid–water 15:5:6 (vol.).

For the detection of formylaspartic acid, formylasparagine and formylisoasparagine the chromatogram was suspended for 1.5 hr. over concentrated hydrochloric acid, ventilated for 16 hr. in the draft of a hood and sprayed with pyridine and ninhydrin solutions successively. The chlorination method as reported[29,30] was not sensitive enough for these three compounds but was used for all other compounds that were not detectable by spraying with ninhydrin solution. The method of Rabinowitz[14] was used in addition to establish the identity of formiminoaspartic acid and methyleneasparagine. The composite chromatogram (Fig. 6) summarizes the chromatographic resolution of the various derivatives of aspartic acid mentioned in this investigation.

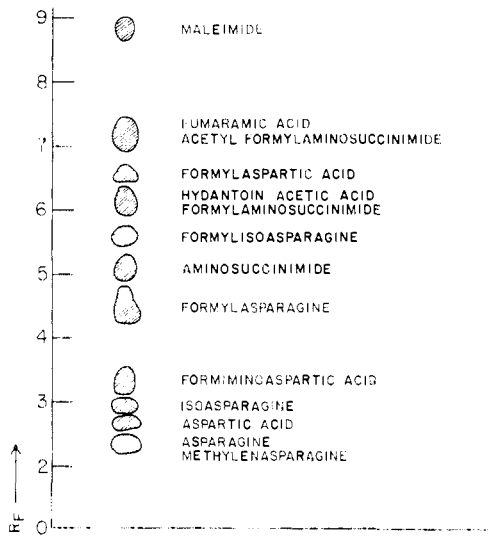


Fig. 6.—Chromatographic resolution of aspartic acid derivatives (Whatman Paper No. 1, butanone:propionic acid:water 15:5:6).

α-Ethyl Formimino-L-aspartate (II).—A solution of 322 mg. (4 mmoles) of formamidine hydrochloride in 3 ml. of formamide was stirred for 2 hr. with 276 mg. (1 mmole) of silver carbonate. The mixture was filtered and the residue washed with 2 ml. of formamide. To this solution was added 323 mg. (2 mmoles) of α-ethyl L-aspartate.[9] The reaction mixture was stirred for 6 hr. at room temperature. The solution was cooled to 0°, diluted with 100 ml. of ice-cold 0.05 *N* hydrochloric acid and chromatographed on a column of Dowex 50-H^+ (8% cross-linked, 200–400 mesh, 3.8 cm.^{2} × 24 cm.) at 3–5° by gradient elution by the addition of 4.0 *N* hydrochloric acid to a mixing reservoir containing 500 ml. of water in such a way that the rates of influx and efflux were equal. α-Ethyl formimino-L-aspartate appeared in the 600–750 ml. fraction and was detected by adjusting the *p*H to 10.5 and observing the ultraviolet absorption peak at 259 mμ characteristic of imidazoloneacetic acid. Attempts to isolate α-ethyl formimino-L-aspartate as hydrochloride or reineckate failed. Lyophilization of an acidic solution of α-ethyl formimino-L-aspartate or of imidazoloneacetic acid gave colored products with ultraviolet spectra different from the original solution. The strongly acidic eluates from the chromatographic column were adjusted to *p*H 3-3.5 by the cautious addition of Dowex 1-OH^- at 2–5° in the cold room. The suspension was filtered and the residue washed with water. The solutions were concentrated to 1/3 of the original volume by lyophilization and stored at −75°. The success of this preparation is dependent on the use of purest reagents and the strict observation of the directions given above.

Rate of Formation of 4(5H)-Imidazolone-5(4)-acetic Acid (III).—From the above solution of α-ethyl formimino-L-aspartate 0.5 ml. was brought to a volume of 2.7 ml. by the addition of water and rapidly mixed with 0.3 ml. of a 1.0 *M* glycine–NaOH buffer of varying *p*H. The rate of formation of III at 26° was followed by measuring the increase of absorption at 259 mμ in a self-recording spectrophotometer. The results are summarized in Fig. 1.

Enzymatic and Non-enzymatic Decomposition of Imidazoloneacetic Acid.—A purified enzyme preparation of imidazoleacetic acid oxidase from *Pseudomonas* (220-fold purification)[4] was kindly placed at our disposal by Dr. O. Hayaishi. To 1.0 ml. of the α-ethyl formiminoaspartate stock solution was added 4.0 ml. of water. The *p*H was adjusted to 10.5–10.8 with 0.1 *N* sodium hydroxide. After 30 seconds 0.6 ml. of buffer solution was added and the solution distributed to two cells of 1 cm. light path. The absorption was measured at 259 mμ. Then 50 λ of the enzyme solution was added to one cell and the decrease of absorption in both cells was measured in intervals of one minute (Fig. 3). Initial rate constants of enzymatic and non-enzymatic degradation are summarized in Fig. 2. Measurements at *p*H <7 were inaccurate because the absorption of the imidazolone at 259 mμ decreased progressively.

Influence of Metal Ions.—Magnesium, manganese and zinc ions in concentrations up to 0.001 *M* failed to influence the rate of spontaneous hydrolysis. The addition of Cu^{++}, Ni^{++}, Co^{++} and Fe^{++} ions caused a marked change over the entire range of the spectrum (200–600 mμ) indicative of rapid complex formation.

Identification of the Products.—To 8 ml. of α-ethyl formimino-L-aspartate stock solution was added enough 0.1 *N* sodium hydroxide to bring the *p*H to 10.5. After 30 sec. the solution was adjusted to *p*H 7.0–7.2 with 0.01 *N* hydrochloric acid. To half of this solution was added 200 λ of enzyme solution and the *p*H was kept constant by the addition of 0.001 *N* sodium hydroxide. After 30 min. the enzyme-containing solution was frozen, lyophilized to about 1 ml. after the addition of some 0.1 *N* hydrochloric acid to bring to *p*H 3 and redissolved. After centrifugation the supernate was neutralized. As a control, the other half of the solution was kept at *p*H 7.0–7.2 without enzyme for 4 hr. Both solutions were chromatographed on paper next to a mixture of reference compounds, *i.e.*, aspartic, formylaspartic formiminoaspartic acids, formylisoasparagine and isoasparagine.

Attempted Syntheses of 5,6-Dihydro-4-pyrimidone-6-carboxylic Acid (VIII).—A. Via Reaction of Formiminoaspartic Acid with Diazomethane.—To a suspension of 10 mg. of formiminoaspartic acid in 0.1 ml. of water was added at 0° 2 ml. of a 0.3 *M* solution of diazomethane in ether. The mixture was shaken for 8 hr. at 0°. The ether was evaporated and the residue treated for 10 hr. with another 2 ml. of diazomethane solution. After the addition of 1 ml. of 0.001 *N* hydrochloric acid the ether was evaporated *in vacuo*. The remaining aqueous solution was diluted to 100

(26) *Cf.* H. Tabor, *Pharmacol. Rev.*, **6**, 299 (1954).

(27) T. W. Beiler, unpublished.

(28) All melting points are corrected, all boiling points uncorrected. The analyses were performed by Dr. W. C. Alford and associates of the Analytical Services Unit of this Laboratory.

(29) H. N. Rydon and P. W. G. Smith, *Nature*, **169**, 922 (1952).

(30) F. Reindel and W. Hoppe, *Naturwissenschaften*, **40**, 221 (1953); *Chem. Ber.*, **87**, 1103 (1954).

ml. with water. Differential spectra were taken by measuring ultraviolet absorption of an alkaline solution (*p*H 8.5) *versus* a reference cell containing an acidic solution (*p*H 3.5) of the reaction products. Two peaks at 257 and 221 mμ were observed, the first belonging to imidazoloneacetic acid (III), the second probably to the dihydropyrimidone VIII.

B. *Via* **Cyclization of β-Methyl Formiminoaspartate.**—Into a suspension of 5 mg. of formiminoaspartic acid in 10 ml. of absolute methanol was bubbled a slow stream of dry hydrogen chloride until after 10 min. a clear solution was obtained. The solvent was removed in a flash evaporator and the residue dissolved in 5 ml. of 0.01 *N* hydrochloric acid at 0°. When the solution of β-methyl formiminoaspartate hydrochloride was taken to dryness, redissolved in a small volume of 0.01 *N* hydrochloric acid at 0° and brought to *p*H 10.5, there was a strong ultraviolet absorption peak at 250 mμ. The precursor of this chromophore was unstable, since it was found that a sample of the material in the 0.01 *N* hydrochloric acid solution after standing for 20 min. and 2 hr. shifted its maximum to 240 mμ and 225 mμ, respectively, after adjustment to *p*H 10.5.

C. **By Hydrogenation of 5-Bromo-4-pyrimidone-6-carboxylic Acid (X): (a) With Palladium-on-Charcoal.**—When 219 mg. of 5-bromo-4-pyrimidone-6-carboxylic acid (X)[19] was hydrogenated in 40 ml. of 50% acetic acid in the presence of 44 mg. of 10% palladium-on-charcoal at room temperature, 2 moles of hydrogen was taken up within 3.5 hr. The mixture was filtered from the catalyst and evaporated *in vacuo* at room temperature to dryness. The residue, a greenish-yellow oil, was chromatographed on paper. Five components were detected and identified as asparagine, methyleneasparagine,[31] aspartic acid, 4-pyrimidone-6-carboxylic acid (IX) and the initial brominated acid (X).

(b) **With Rhodium on Alumina.**—When 318 mg. of 5-bromo-4-pyrimidone-6-carboxylic acid (X) in 35 ml. of acetic acid was hydrogenated with 100 mg. of 5% rhodium catalyst on alumina, the slow uptake of hydrogen came to a stop after 18 hr. and an uptake of 0.9 mole of hydrogen. Examination of the reaction mixture by paper chromatography showed the same components as above in about the same proportions.

D. **By Hydrogenation of 4-Pyrimidone-6-carboxylic Acid (IX).**—When 28 mg. of the acid IX dissolved in 20 ml. of water was hydrogenated with 31 mg. of 5% rhodium catalyst on alumina, 1 mole of hydrogen was consumed within 4.5 hr. The reduction mixture, when chromatographed as above, showed about the same composition. In another experiment 0.25 mg. of the acid IX in 3 ml. of 0.1 *N* hydrochloric acid in glacial acetic acid was hydrogenated with 4.5 mg. of 5% rhodium catalyst on alumina in a Warburg apparatus; 0.28 mole of hydrogen was taken up after 19 hr. at 28°. The reaction mixture contained formiminoaspartic acid[10] identified on paper chromatograms (R_{FII} 0.33) by Rydon's method[28,29] or with the more specific reagent of Rabinowitz.[15] Parallel runs in acetic acid and 50% ethanol did not show any trace of formiminoaspartic acid. No hydrogenation occurred in dimethylformamide, N-ethylmorpholine and dimethyl sulfoxide.

Formylaminosuccinimide (XVII). A. By Dehydration of Formylasparagine.—A solution of 1.6 g. (10 mmoles) of formylasparagine (XV) in 20 ml. of acetic anhydride was slowly concentrated at atmospheric pressure in an open flask until after 1 hr. the vapor temperature reached 140° and the solution was concentrated to about 8 ml. The brown solution was evaporated *in vacuo*. The oily residue, when dissolved in 2 ml. of ethanol, yielded 0.6 g. of colorless crystals which after two additional recrystallizations had m.p. 180–182°. R_F 0.38 (solvent system I); 0.62 (solvent system II).

An additional labile substance was present in the mother liquor. It had R_{FI} 0.48, R_{FII} 0.72 and, on extraction from paper and rechromatography, was converted to formylaminosuccinimide (XVII).

B. **By Sublimation of Formylasparagine.**—When formylasparagine was sublimed at 180° and 10^{-3}–10^{-4} mm., formylaminosuccinimide was formed directly as a colorless, analytically pure sublimate.

Anal. Calcd. for $C_5H_6N_2O_3$: C, 42.25; H, 4.26; N, 19.71. Found: C, 42.34; H, 4.08; N, 19.72.

(31) H. Schiff, *Ann.*, **310**, 30 (1900).

Aminosuccinimide (XVIII).—A solution of 1.0 g. of formylaminosuccinimide (XVII) in 50 ml. of 1.5 *N* absolute methanolic hydrogen chloride was left for 18 hr. at room temperature. After evaporation of the solvent *in vacuo* the residue was recrystallized from ethanol–ether. The colorless crystals of the hydrochloride had m.p. 222–223° dec. A solution of 100 mg. of this hydrochloride in 5 ml. of water was treated with Dowex I (OH^- form) and the residual sirup set aside with one drop of ethanol. The crystals which formed on standing were washed with ethanol–ether. They had m.p. 140–143°. After an additional crystallization from dimethylformamide–ether, the m.p. rose to 143–145°, undepressed on admixture with aminosuccinimide prepared by the method of Sondheimer and Holley.[21] The R_F in the solvent system II was 0.50. It gave a brownish-yellow color with ninhydrin and showed a strong yellow fluorescence under ultraviolet light.

Carbobenzyloxyaminosuccinimide (XIX).—To 450 mg. of aminosuccinimide hydrochloride and 500 mg. of a suspension of magnesium oxide in 10 ml. of water and 5 ml. of ether was added dropwise at 0° 1.3 ml. of carbobenzyloxychloride within 10 min. The mixture was stirred for 2 hr. at 0°, filtered, separated from the ether layer and extracted with two additional portions of ether. The aqueous layer was acidified with hydrochloric acid and extracted with 3 portions of ethyl acetate. After evaporation 673 mg. of a gum was left which crystallized slowly within 2 days. After two recrystallizations from ethyl acetate–hexane the m.p. was 78–81° (reported 79–81°[21]), undepressed on admixture with carbobenzyloxyaminosuccinimide prepared according to Sondheimer and Holley.[21]

Anal. Calcd. for $C_{12}H_{12}N_2O_4$: C, 58.06; H, 4.87; N, 11.29. Found: C, 58.01; H, 4.71; N, 11.12.

Isoasparagine (V).—Carbobenzyloxyisoasparagine was prepared from α-ethyl carbobenzyloxyaspartate.[9] It melted at 167–169° after one crystallization from water. Catalytic decarbobenzyloxylation gave chromatographically pure isoasparagine uncontaminated by asparagine or aspartic acid. By contrast, the opening of carbobenzyloxyaspartic anhydride with aqueous ammonia[32] or of phthaloylaspartic anhydride with alcoholic ammonia[33] and subsequent removal of the N-protecting group did not give pure samples of isoasparagine.[34]

Formylisoasparagine (IV).—When 100 mg. of isoasparagine (V) was kept for 2 hr. at 35° in a mixture of 5 ml. of formic acid (98%) and 5 ml. of acetic anhydride, the solution evaporated *in vacuo* to dryness and the residue dissolved in 2 ml. of ethanol, the formylisoasparagine could be precipitated as a gum by the addition of 2 ml. of ether. The product crystallized after 3 days and was recrystallized twice from ethanol–ether. There was obtained 56 mg. of colorless crystals, m.p. 133–135°, R_{FII} 0.56.

Anal. Calcd. for $C_5H_8N_2O_4$: C, 37.50; H, 5.04; N, 17.50. Found: C, 37.79; H, 5.26; N, 17.66.

Acetylation of Formylaminosuccinimide with Ketene.—Through a suspension of 142 mg. of formylaminosuccinimide and 83 mg. of fused sodium acetate in 5 ml. of boiling carbon tetrachloride was bubbled a slow stream of ketene.[35] After 1 hr. the hot suspension was filtered and the residue washed with 2 ml. of hot carbon tetrachloride. The filtrate, evaporated to dryness, yielded 71 mg. of a brown oil. Chromatograms showed 3 substances: R_{FII} 0.63, 0.79 and 0.85. Only one, R_{FII} 0.63 with starting material gave, after treatment of the papers with hydrochloric acid the yellow fluorescent spot with ninhydrin, typical of aminosuccinimide.

From acetylation without sodium acetate 90% of crystalline starting material was recovered and the only other detectable product, a Rydon-positive substance (R_{FII} 0.79), did not give aminosuccinimide on mild acid hydrolysis.

BETHESDA 14, MD.

(32) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

(33) S. W. Tanenbaum, THIS JOURNAL, **75**, 1754 (1953).

(34) *Cf.* W. B. Lutz, C. Ressler, D. E. Nettleton, Jr., and V. du Vigneaud, *ibid.*, **81**, 167 (1959).

(35) *Cf.* R. F. Dunbar and M. Swanson, *J. Org. Chem.*, **23**, 179 (1958).