Autoxidation of Diphenylacetaldehyde *p*-Methoxyanil.—When 3 g. of the Schiff base was dissolved in 20 ml. of ethyl acetate, 235 ml. of oxygen was taken up within 20 minutes when the solution was agitated in a closed system under oxygen. The oxidized solution gave a hydroperoxide test which was negative after 2 hr. of standing at room temperature. On concentration of the solution and addition of petroleum ether, rosettes of short rectangular rods were obtained, m.p. 80–82°, identical with *p*-formanisidide. The petroleum ether mother liquors yielded benzophenone, characterized and identified by infrared comparison.

Dehydrobufotenine Hydrochloride (XLVIII).—Thirty milligrams of dehydrobufotenine picrate[53] was dissolved in 3 ml. of 2 *N* hydrochloric acid and extracted with ethyl acetate until the aqueous phase was free of picric acid.[54]

The hydrochloride crystallized on concentration in the desiccator from the aqueous acid in long colorless needles, losing their transparency above 100°, charring at 215° and progressively darkening and decomposing, no melting up to 300°.

Dihydroberberine (XXXIX).—Following the procedure of Freund and Fleischer[31] the free dihydroberberine, prepared from the yellow-red hydrochloride from the filtrate of oxoberberine, was recrystallized from benzene and obtained in the form of yellow prisms with a green tinge, m.p. 157–159° (discoloration and decomposition starting at 146°).

(53) The sample came from Prof. V. Deulofeu, Buenos Aires, and was obtained through the courtesy of Dr. M. E. Speeter, The Upjohn Co., Kalamazoo, Michigan.

(54) *Cf.* the conversion of bufotenidine picrate into the hydrochloride: H. Wieland, W. Konz and H. Mittasch, *Ann.*, **513**, 18 (1934).

BETHESDA 14, MD.

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, AND THE DEPARTMENT OF CHEMISTRY, STETSON UNIVERSITY]

The Conversion of L-Histidine into Hydroxy- and Allohydroxy-proline *via erythro-* and *threo*-γ-Hydroxy-L-ornithine[1,2]

BY BERNHARD WITKOP AND THEODORE BEILER[2]

RECEIVED DECEMBER 27, 1955

α,δ-Dibenzoyl-γ-keto-L-ornithine methyl ester (IV), obtainable from L-histidine methyl ester (I) by Bamberger cleavage, on hydrogenation yielded the two diastereoisomeric lactones (VIII and IX) of *erythro* and *threo*-α,δ-dihexahydrobenzoyl-γ-hydroxy-L-ornithine. Hydrolysis furnished the dihydrochlorides of the two diastereoisomeric lactones (X and XIII) of γ-hydroxy-L-ornithine accompanied in each case by some of the D-isomers (XI and XII) arising by acid catalyzed epimerization at C(2). The various γ-hydroxyornithines were assayed and separated by chromatography on Dowex-50 columns. The mutarotation of these lactones in aqueous solution parallels the ring opening which was followed by measuring the carbon dioxide produced by reaction with Chloramine T. The reaction of the various lactones with nitrosyl chloride, before and after mutarotation, and subsequent base-catalyzed cyclization of the mixture of α- and δ-chlorohydroxyamino acids yielded mixtures of normal and allohydroxyproline (XIV and XV; XVI and XVII) which were partially fractionated *via* their remarkably different reineckates, analyzed by Dowex-50 and identified by their rotations. These transformations made possible the stereochemical correlation of C(4) in *erythro*-γ-hydroxy-L-ornithine (X) with C(4) in hydroxy-L-proline (XIV) and in *threo*-γ-hydroxy-L-ornithine (XIII) with allohydroxy-L-proline (XVII). Preliminary results on the transamination of γ-hydroxyornithine preparations are reported.

γ-Hydroxyornithine, though not isolated from natural sources, can be visualized as a precursor or labile metabolite of hydroxyproline[3] or as a building stone in the biogenesis of scopolamine,[4] and several synthetic attempts, leading to racemic mixtures of undefined purity, have been recorded in the literature.[5–9] This paper describes the synthesis of the two diastereoisomers of γ-hydroxy-L-ornithine from L-histidine and the establishment of the stereochemistry of C(4), to which the secondary hydroxyl group is attached, by conversion to (allo)-hydroxyproline.[10]

Separation and Properties of the Two Diastereoisomeric Dihexahydrobenzoyl-γ-hydroxy-L-ornithine Lactones.—The catalytic reduction (platinum in acetic acid or Raney nickel in methanol) of α,δ-dibenzoyl-γ-keto-L-ornithine (IV),[8] obtainable *via* Bamberger cleavage[11] of L-histidine methyl ester (I)[12–14] led to the continuous uptake of seven moles of hydrogen with no indication of the γ-keto group being reduced prior to reduction of the benzene rings.[15] Even the use of Raney nickel in methanol

(1) Labile Metabolites. III. Preceding paper in this series, THIS JOURNAL, **76**, 5579 (1954).

(2) Presented in part at the Meeting of the Chemical Society (London) on Natural Heterocyclic Compounds held at Exeter, England, July 13–15, 1955; *cf.* Special Publication No. 3, The Chemical Society, Burlington House, W. 1, London, 1955, pp. 60–82.

(3) Possible pathways for the biosynthesis of γ-hydroxyornithine are the transamination of γ-hydroxyglutamic semialdehyde, a metabolite of hydroxyproline [K. Lang and U. Mayer, *Biochem. Z.*, **324**, 237 (1953)] or the reduction of a hypothetical keto-ornithine, formed possibly by the condensation of aspartate with glycine in analogy to the formation of δ-aminolevulinic acid from succinate and glycine [D. Shemin and C. S. Russell, THIS JOURNAL, **75**, 4873 (1953). Dihydroxyornithine and its betaine, myokinine, are of doubtful occurrence [R. Engeland and A. Bastian, *Compt. rend.*, **207**, 945 (1938)].

(4) E. Lecte, L. Marion and I. D. Spenser, *Can. J. Chem.*, **32**, 1116 (1954).

(5) E. Hammarsten, *Compt. rend. trav. lab. Carlsberg*, **11**, 223 (1916).

(6) W. Traube, R. Johow and W. Tepohl, *Ber.*, **56**, 1861 (1923).

(7) M. Tomita and T. Fukagawa, *Z. physiol. Chem.*, **158**, 58 (1926).

(8) W. Langenbeck and R. Hutschenreuter, *ibid.*, **182**, 305 (1929).

(9) A. N. Dey, *J. Chem. Soc.*, 1166 (1937).

(10) *Cf.* I. Uematsu, H. Ando and M. Uchida Seikagaku, *J. Biochem., Japan*, **26**, 386 (1954).

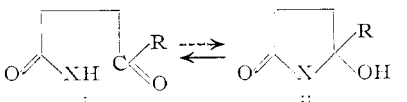
(11) *Cf.* E. Bamberger and O. Berlé, *Ann.*, **273**, 342 (1893).

(12) A. Kossel and F. Edlbacher, *Z. physiol. Chem.*, **93**, 396 (1914); A. Windaus, *et al.*, *Ber.*, **43**, 499 (1910).

(13) J. N. Ashley and C. R. Harington, *J. Chem. Soc.*, 2586 (1930); C. R. Harington and J. Overhoff, *Biochem. J.*, **27**, 338 (1933).

(14) H. Heath, A. Lawson and C. Rimington, *J. Chem. Soc.*, 2215 (1951).

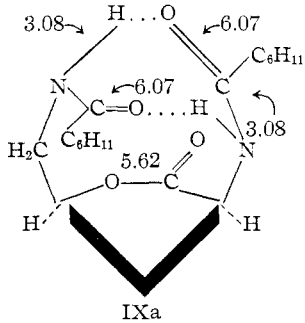
(15) An equilibrium of ring-chain tautomers, i ⟶ ii, may be considered for γ-keto compounds such as the two forms of ketoglutaramic



acid [i ⟶ ii: X = NH, R = COOH; *cf.*, A. Meister, *J. Biol. Chem.*, **210**, 17 (1954)] or the γ ketoacid from furan [i ⟶ ii: X = O; R =

gave complete reduction and only minor amounts of the dibenzoyl (V) or of the two position-isomeric benzoylhexahydrobenzoyl lactones (VI) (Table III, Experimental). The major product in reductions with platinum or nickel was the dihexahydrobenzoyl lactone VII which betrayed its inhomogeneous character only after repeated and careful recrystallizations. Two pure lactones, m.p. 242–243° and 262.5–263° (VIII and IX), were obtained in this way. Compared with the original mixture or fractions from mother liquors, they showed the properties summarized in Table IV (see Experimental).

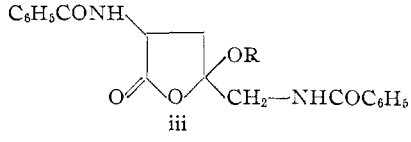
The infrared spectra are shown in Fig. 1. The reversible mutarotation and the presence of only one amide carbonyl in the infrared spectrum of IX indicate the existence of hydrogen bridges and their rupture by strong acid. Strainless intramolecular hydrogen bridges can be constructed with Stuart–Briegleb models only in the *cis*-series. The structure of a *cis*-disubstituted lactone IXa may be tentatively adopted for the lactone, m.p. 263°, and the



IXa

(non-bonded) *trans*-structure VIII for the lactone, m.p. 243°. Both lactones are formed in roughly equal amounts in the reduction with platinum, while only 10% low melting isomer is isolated in the reduction with Raney nickel (Table III).[16] A significant difference between the two lactones is their behavior on acid hydrolysis. The higher melting lactone is distinctly more difficult to hydrolyze.

CH_2–CH_2–NH_2; O. Moldenhauer, W. Irion, D. Mastaglio, R. Pfluger and H. Döser, *Ann.*, **583**, 50 (1953); C. Grundmann and W. Ruske, *Ber.*, **86**, 939 (1953)] in order to explain the lack of carbonyl reactivity in these compounds. α,δ-Dibenzoyl-γ-keto-L-ornithine, obtainable from its methyl ester by base hydrolysis, showed only a single CO band in the infrared, giving back, on re-esterification with diazomethane, the original ester. The existence of tautomeric and diastereoisomeric forms, iii (R = H or CH_3), has not been rigidly excluded by



iii

chemical or infrared data; lactols absorb at 5.72μ [J. F. Grove and H. A. Willis, *J. Chem. Soc.*, 877 (1951)], IV absorbs at 5.72 μ (Nujol). It is significant that our attempts to esterify or to reduce with sodium borohydride γ-keto-L-ornithine and its derivatives were without success.

(16) The introduction of a second center of asymmetry by the reduction of an unsaturated γ-lactone has been shown to lead to the formation of only the *trans*-diastereoisomer [H. Schinz, *et al.*, *Helv. Chim. Acta*, **33**, 130, 140 (1950)]. In the reduction of III the stereochemical course may depend on the possible presence of diastereoisomeric lactols (footnote 15) and the stabilizing influence of hydrogen bonding in the latter as well as in the reduction products.

The Preparation and Mutarotation of the Two Diastereoisomeric γ-Hydroxyornithine Lactone Dihydrochlorides.—Acid hydrolysis of the two dihexahydrobenzoyl lactones by conventional methods (6 *N* HCl for 3 hours and more) yielded the free lactone dihydrochlorides as hygroscopic gummy residues which became crystalline on prolonged trituration with ethanol but had no distinctive melting points. Recrystallization was impossible, and the free lactones were unstable. The polarimetric constants served as the only guide for the characterization or differentiation of various preparations (Table V, Experimental).

The nature of the mutarotation of these lactones became apparent when it was found that the rate of evolution of carbon dioxide in the reaction of γ-hydroxyornithine with Chloramine T at various stages of equilibration in water paralleled the rate of mutarotation (Table I).

TABLE I

RATE OF RING OPENING IN γ-HYDROXYORNITHINE LACTONE BASED ON EVOLUTION OF CARBON DIOXIDE BY REACTION WITH CHLORAMINE T AT *p*H 4.7 AND 25°

	Compound	Time of equilibration in H_2O (hr.)	% CO_2 after 6 min.
A	Ornithine	..	100
B	Hydroxyornithine[a]	..	100
C	Hydroxyornithine lactone	1	30
D	Hydroxyornithine lactone	20	60
E	Hydroxyornithine lactone	96	90

[a] Sodium salt reacted immediately after acidification.

Free hydroxyornithine, prepared by acidification of the sodium salt, behaves like ornithine and yields 100% of carbon dioxide in the reaction with Chloramine T.

The Separation of the Two Diastereoisomeric γ-Hydroxyornithines on Ion-exchange Resin.—It was suspected that in the course of acid hydrolysis partial epimerization had occurred, but there was no separation of the two lactone dihydrochlorides on paper (solvent mixture: formic acid (15%), water (15%), *t*-butyl alcohol (70%); pyridine and phenol combinations gave no discrete spots).

After the successful separation of the diastereoisomers of isoleucine, hydroxylysine and hydroxyproline on Dowex-50[17] we tried this technique on the hydrolysates from VIII and IX. As a marker we used "natural" L-δ-hydroxylysine, obtainable by acid hydrolysis of collagen. Such hydroxylysine preparations contain about 18% allohydroxy-D-lysine,[18] depending on the duration of acid hydrolysis. There can be little doubt that the epimerization occurs at C(2). This easy epimerization with acid is only found with certain hydroxyamino acids and will occur only at the stage of the γ- or δ-lactone which in the case of hydroxylysine is not isolated. Figure 2 demonstrates the separation of the two hydrolysates from VIII and IX. They are mixtures and show that three hours' refluxing with 6 *N* HCl effected 30% epimerization, presumably at C(2). When the refluxing time is shortened to 45 minutes

(17) K. A. Piez, *J. Biol. Chem.*, **207**, 77 (1954).

(18) P. B. Hamilton and R. A. Anderson, *ibid.*, **213**, 249 (1955). We feel greatly indebted to Dr. Hamilton for samples as well as for letting us see his manuscript prior to publication.

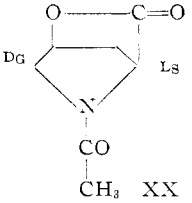
preparations are obtained which according to the column assay are 90 ± 10% pure.

In order to avoid acid hydrolysis the Bamberger cleavage of histidine methyl ester with carbobenzyloxy chloride was tried; it led to an unstable monocarbobenzyloxy compound (m.p. 109–112°) considered to be carbobenzyloxy-L-histidine methyl ester.[19]

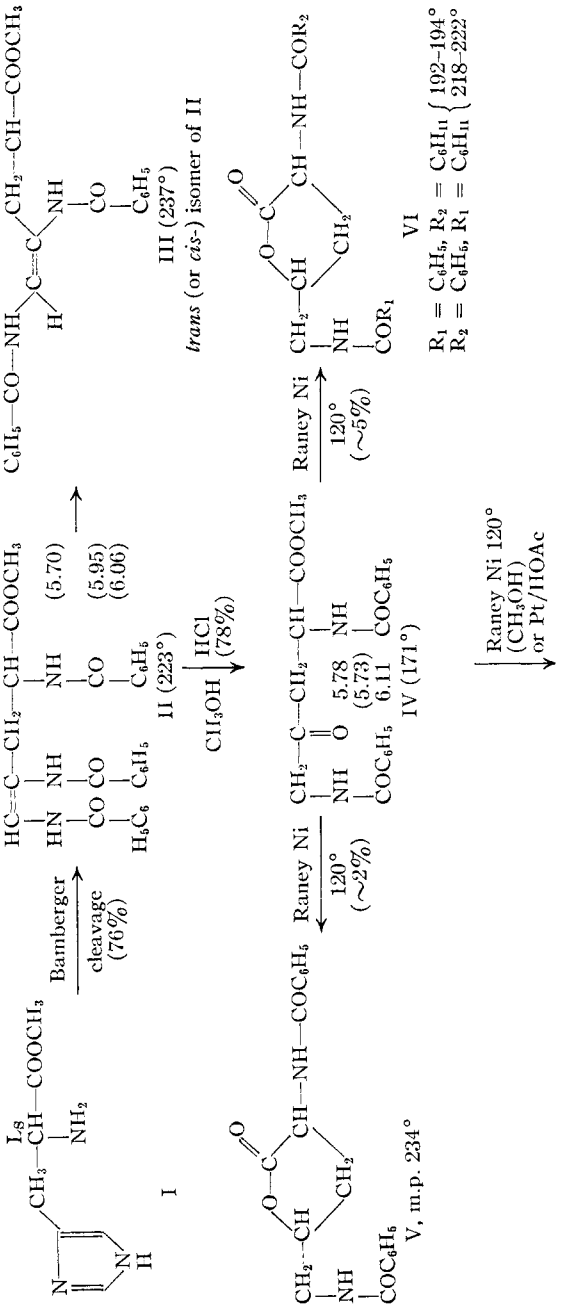
The Conversion of Hydroxyornithine to (*allo*) Hydroxyproline and their Separation.—So far no binding stereochemical correlations have been made. On the basis of the infrared spectra the *trans*-structure VIII was favored for the lactone, m.p. 243° and the *cis*-arrangement IX for the lactone, m.p. 263°. Chart II shows that a *trans*-lactone opens up to L-*erythro*-γ-hydroxyornithine with the L_S configuration at C(2) and L_G configuration[20] at C(4).

Conversely, the *cis*-lactone will give L-*threo*-γ-hydroxyornithine. Both compounds must be in the natural amino acid or L_S-series since L-histidine was used as the starting material and no racemization or epimerization could have occurred at any intermediate step. On hydrogenation a second center of asymmetry was introduced at C(4) and the diastereoisomers VIII and IX were formed. Any knowledge about the configuration at C(4) would give the complete stereochemistry of VIII and IX.

Neuberger[21] has correctly attributed the "d-configuration" to C(4) in natural L-hydroxyproline. If one translates these correlations into the accepted carbohydrate nomenclature using capital letters L_G and D_G, L-(+)-methoxysuccindiamide (dextrorotatory methoxysuccindiamide from 4-methoxy-L-proline[20]) does not assume a D_G- sign until the four carbon chain becomes a three carbon chain, *viz.*, D-(−)lactic acid and D-(+)-glyceraldehyde. The proper assignment of configuration for C(4) in L-hydroxyproline in the unambiguous terms of carbohydrate nomenclature, therefore, is L_G. This clarification is needed for the evaluation of the transformation of hydroxyornithine to hydroxyproline and in view of other interpretations in the literature.[22] The use of carbohydrate nomenclature is justified and this conclusion is strengthened by the finding that the lactone of N-acetylallohydroxy-L-proline (XX), m.p. 99–101°,[23] $[\alpha]^{20}D$ +61.1°, shows a positive difference of rotation of +152.6° as compared with N-acetylallohydroxy-L-proline,



XX



(19) Carbobenzyloxy-L-histidine melts at 209° [M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932)].

(20) *Cf.* H. B. Vickery, *J. Biol. Chem.*, **169**, 242 (1947); *Chem. Eng. News*, **25**, 1365 (1947).

(21) A. Neuberger, *J. Chem. Soc.*, 429 (1945); C. S. Hudson and A. Neuberger, *J. Org. Chem.*, **15**, 24 (1950).

(22) The configuration of C(4) in hydroxy-L-proline is then the same (L_G) as in allo-L-threonine, and conversely, C(4) in allohydroxy-L-proline corresponds to C(3) in L-threonine which is D_G.

(23) A. A. Patchett and B. Witkop, in preparation.

$[\alpha]^{20}D$ −91.5°. Assuming that Hudson's rule is applicable in this particular case[24] this positive difference would indicate the D_G configuration for C(4) in *allo*-hydroxy-L-proline.

In the *in vitro* conversion to hydroxyproline the hydroxyornithine hydrolysates from VIII and IX

(24) *Cf.* W. Klyne, *Chemistry and Industry*, 1198 (1954).

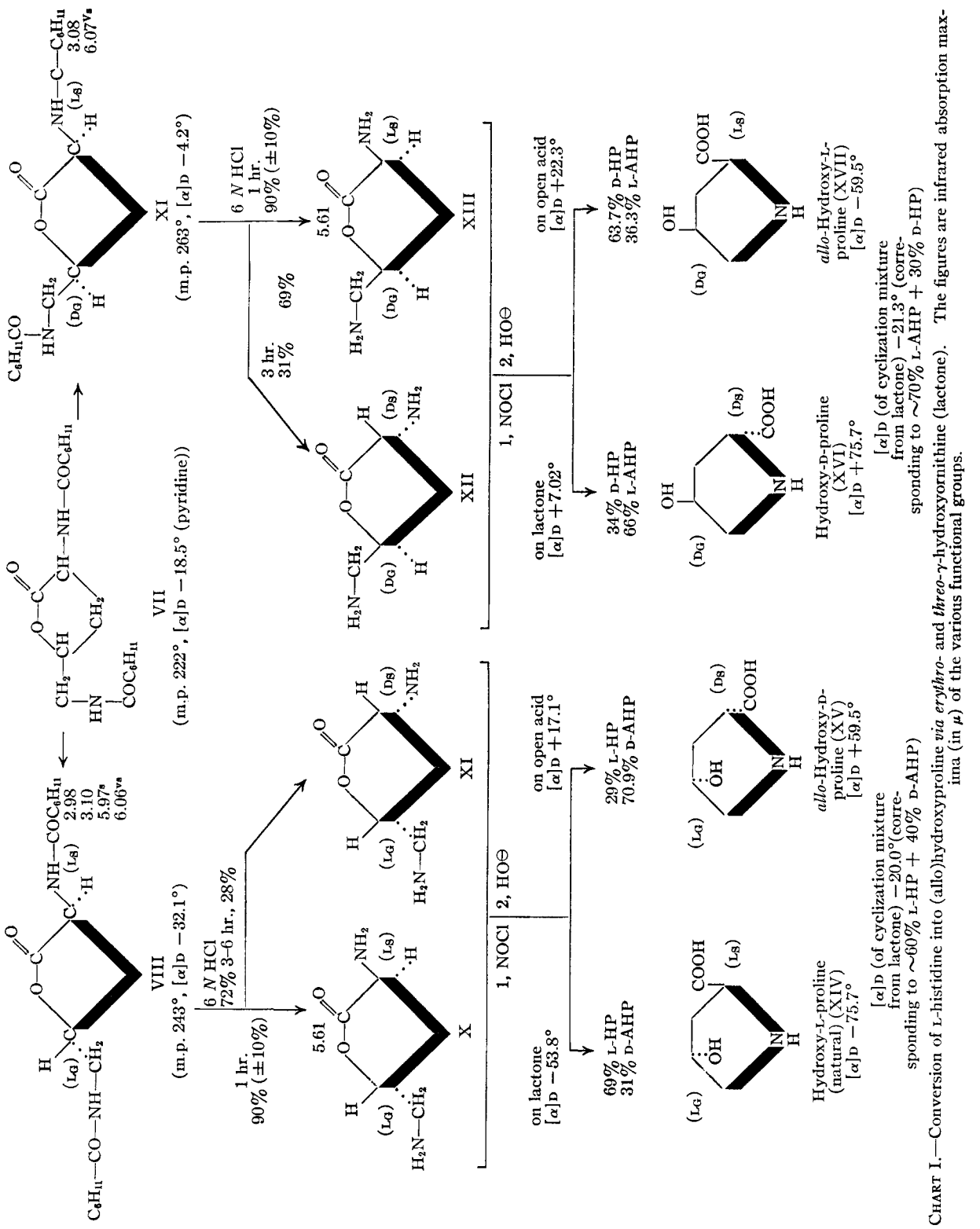


CHART I.—Conversion of L-histidine into (allo)hydroxyproline *via erythro-* and *threo-*γ-hydroxyornithine (lactone). The figures are infrared absorption maxima (in μ) of the various functional groups.

were converted into the mixture of α- and δ-chloroacids and the pyrrolidine rings formed by the action of base.[25] These transformations, regardless of mechanism, do not affect the stereochemistry of C(4). Figure 3 shows the results and methods for separation and Chart I pictures the entire sequence of reactions starting with L-histidine. Additional independent evidence for the *cis-* and *trans*-assignments in VIII and IX will come from an X-ray analysis of the two diastereoisomeric α,δ-di-*p*-iodobenzoyl-γ-hydroxyornithine lactones which is in progress. Alkyl–oxygen fission in the course of mutarotation has been assumed not to play a significant

(25) *Cf.* P. B. Hamilton, *J. Biol. Chem.*, **198**, 587 (1952).

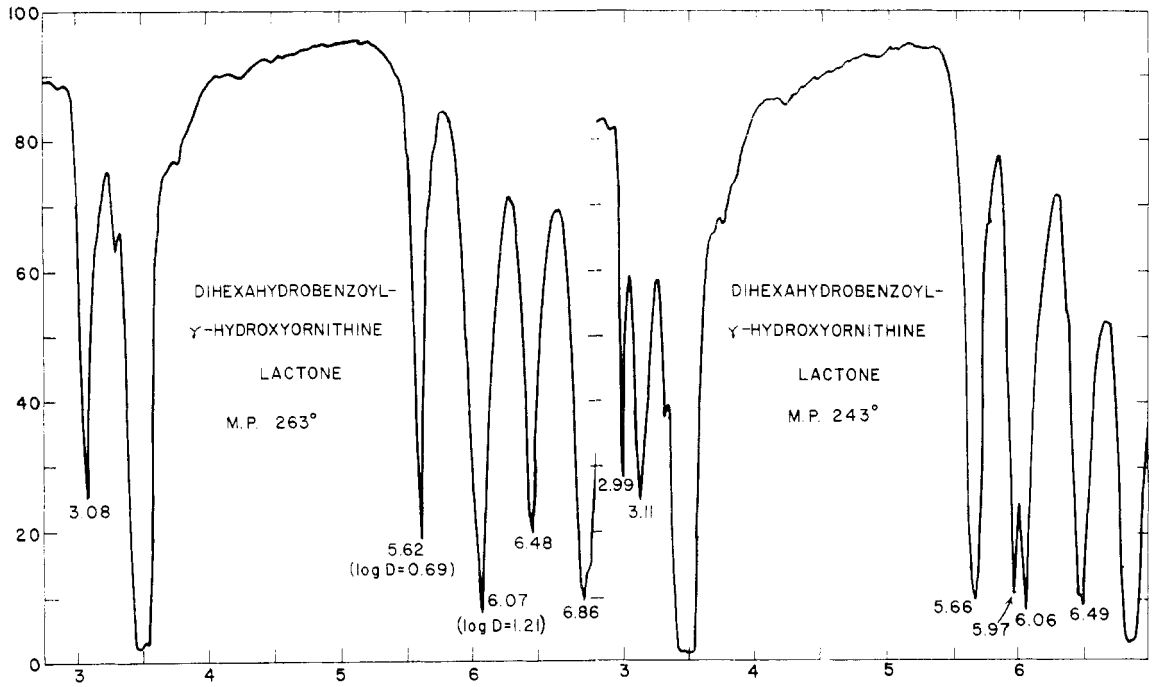


Fig. 1.—Infrared spectra of the two epimeric N,N-dihexahydrobenzoyl-γ-hydroxyornithine lactones in Nujol. The lactone carbonyl on the left has only about half the intensity of the combined lactam carbonyls, whereas on the right the three carbonyls are of approximately equal intensity.

part. As required by studies of models L-*erythro*-γ-hydroxyornithine yields L-hydroxyproline when no center of asymmetry is inverted (cyclization of a δ-chloro intermediate) and allo-hydroxy-D-proline when C(2) is inverted. The inversion of ratios of the two hydroxyproline mixtures *before* and *after* mutarotation of the hydroxyornithine lactones (Table II) reflects on the mechanism of the reaction of nitrous acid with the α-amino group in open amino acid compared with the lactone. It may pro-

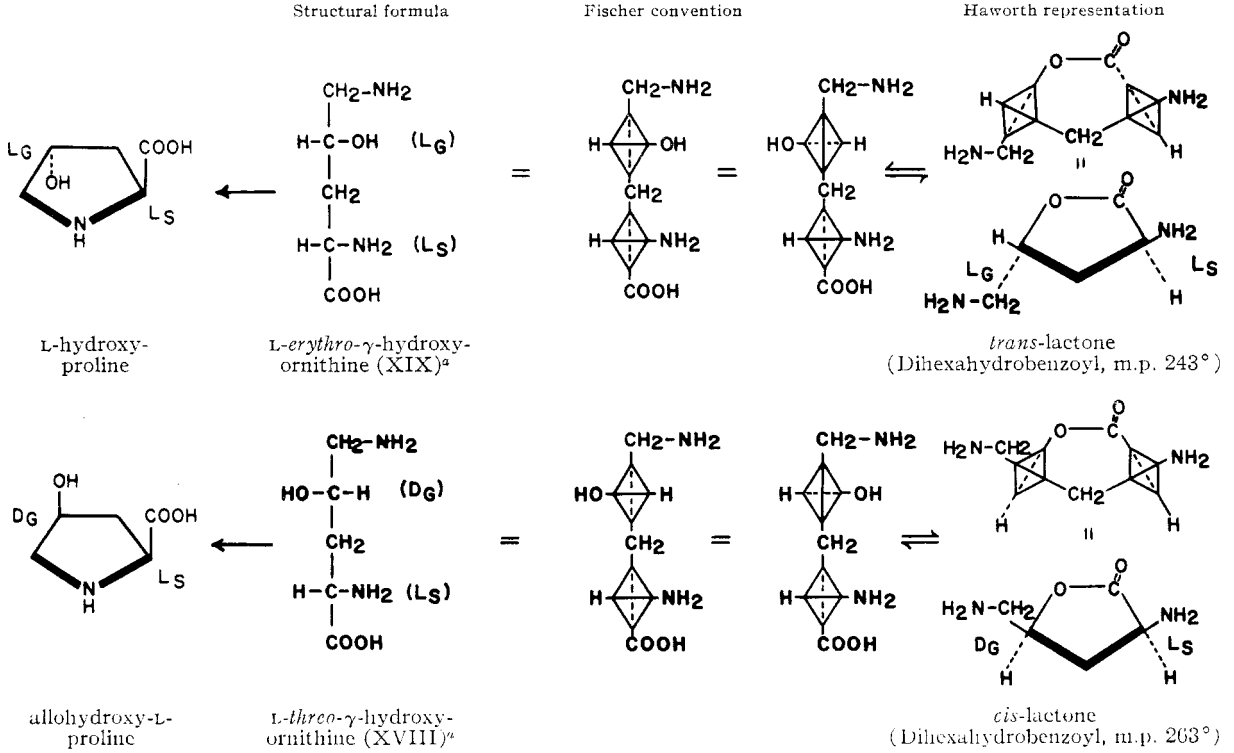


CHART II

[a] The reference point for this nomenclature is carbon 2 which in both cases has the natural L_S-configuration.

TABLE II

RATIOS OF NORMAL AND ALLOHYDROXYPROLINES FROM THE BASE-CATALYZED CYCLIZATION OF THE α- AND δ-CHLOROACIDS OBTAINED BY THE ACTION OF NITROSYL CHLORIDE ON THE VARIOUS γ-HYDROXYORNITHINE MIXTURES (X + XI; XII + XIII) FROM THE TWO PURE DIHEXAHYDROBENZOYL-γ-HYDROXY-L-ORNITHINE LACTONES (VIII, IX) BEFORE AND AFTER MUTAROTATION

From lactone m.p.	Time of hydrolysis in 6 *N* HCl (hr.)	Assay by Dowex	Reaction with nitrosyl chloride: Temp., °C.	Time, min.	Color	Base	Cyclization conditions: Temp., °C.	Time, min.	Hydroxyprolines, %: Yield	Normal	Allo
242°	3	72% L-*erythro*-γ-hydroxyornithine; 28% D-*threo*-γ-hydroxyornithine	60	7	None	*p*H 8	25	48 hr.	12	64	36
	3		55	18	Red	*p*H 10	55	10	59	71	29
	3		20	27	None	*p*H 10	55	10	32	72	28
	3[a]		55	18	None	*p*H 10	55	10	41	29	71
	3/4	90% ± 10% L-*erythro*-γ-hydroxyornithine	55	10	None	*p*H 10	55	10	39	50.7	49.3
263°	5[a]	68% L-*threo*-γ-hydroxyornithine; 32% D-*erythro*-γ-hydroxyornithine	55	18	None	*p*H 10	55	10	44	64	36
	5		55	15	Red	0.2 *N* $Ba(OH)_2$	100	10	17	34	66
	5		55	20	Red	*p*H	60	10	54	33	67
	3/4	90% ± 10% L-*threo*-γ-hydroxyornithine	55	10	Red	*p*H 10	55	10	45	50.2	49.8

[a] Mutarotated.

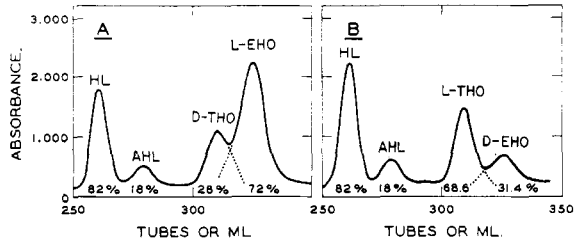


Fig. 2.—Analysis of the epimerization mixtures of γ-hydroxyornithine lactones obtained on acid hydrolysis (3 hours refluxing with 6 *N* HCl) of the two epimeric dihexahydrobenzoyl-γ-hydroxyornithine lactones VIII, m.p. 243°, shown in A, and IX, m.p. 263°, shown in B. The separation of the two epimeric hydroxy-ornithines D-*threo*- and L-*erythro*- and L-*threo*- and D-*erythro*-γ-hydroxyornithines (D-THO and L-EHO; L-THO and D-EHO) in the two mixtures A and B, after mutarotation in phosphate buffer *p*H 7.8, was effected by a 100 cm. long Dowex 50 column, diameter 0.9 cm., at 37° using 0.1 *M* phosphate buffer of *p*H 7.8. The marker was "natural" hydroxylysine, as obtainable from gelatin by acid hydrolysis, containing 82% normal (HL) and 18% *allo*-hydroxylysine (AHL).

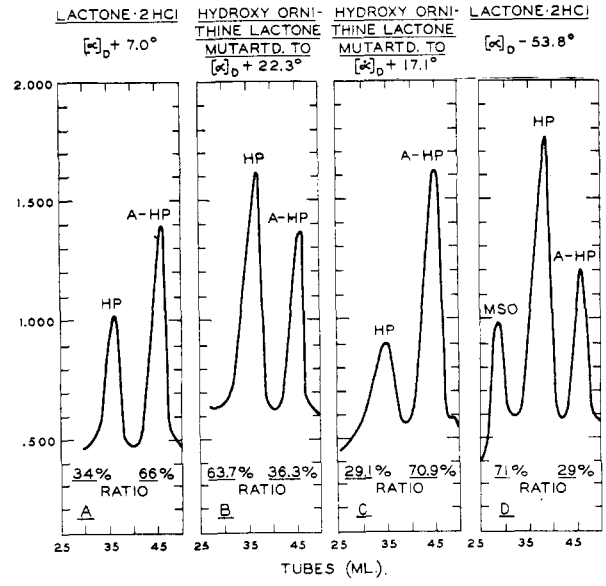


Fig. 3.—Ratios of hydroxyprolines (HP) and *allo*-hydroxyprolines (A-HP) obtained from the two mixtures of *erythro*- and *threo*-γ-hydroxyornithine on treatment with nitrosyl chloride *before* (A, D) and *after* mutarotation in water (B, C) followed by base-catalyzed ring closure of the non-isolated chloro acids. The column was 40 cm. long, had a diameter of 0.9 cm., and was thermostated to 37°; the buffer was sodium citrate, *p*H 3.20. The ordinates show absorbance of λ_{max} 440 mμ for HP and A-HP and of λ_{max} 570 mμ for methionine sulfoxide (MSO) which in each case (shown here only for D) was used as a marker.

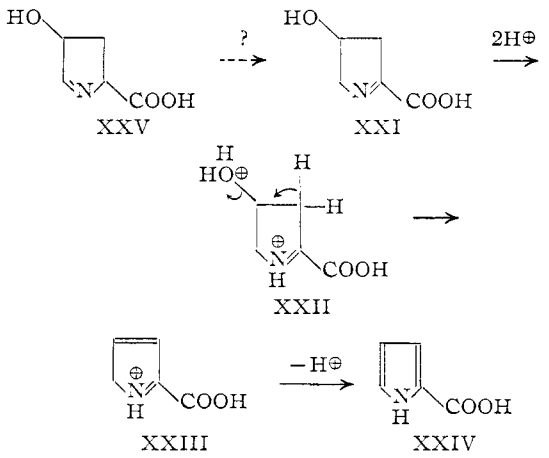
ceed with retention or formation of the epoxide in the open series; whereas the amino lactone may show Walden inversion (or formation of olefin) with nitrous acid depending on the conformation of the amino group.[26] Further investigation will be needed in this field.

Preliminary transamination tests of hydroxyornithine were kindly performed through the courtesy of Dr. A. Meister. The γ-hydroxyornithine lactone dihydrochlorides from VIII and IX were equilibrated in buffer *p*H 7.5 for two days and then incubated with sodium glyoxylate with and without rat liver preparation. The amount of glycine formed from glyoxylic acid with either VIII or IX was noticeably higher in the enzymatic experiments compared with the non-enzymatic control tests and was somewhat smaller than in control experiments with L-ornithine. No transamination occurred with sodium pyruvate or ketoglutarate.

The product to be expected by α-transamination would be dehydrohydroxyproline XXI which is known to undergo facile acid-catalyzed (XXII) β-elimination to form pyrrole-α-carboxylic acid (XXIV) *via* XXII and XXIII.[26a] The analysis of γ-hydroxyornithine transamination mixtures for the presence of XXIV is in progress. If hydroxyproline can be formed from free proline by introduction of a

(26) *Cf.* A. K. Bose, *Experientia*, **9**, 256 (1953).

(26a) A. N. Radhakrishnan and A. Meister, *Federation Proceedings*, **15**, 333 (1956) (ADDED IN PROOF).



hydroxyl in C(4) at the dehydroproline stage, the stability of the intermediate XXV would depend on the ease of isomerization to XXI.

Experimental[27]

Methyl 2,4,5-Tribenzamido-Δ^{-4}-L-pentoate (II).—The procedure of Heath, Lawson and Rimington[14] was followed with some modifications. The crude yield was 37.1 g. (76% yield), m.p. 213–216°. Two recrystallizations from methanol gave material melting at 222–223°. The pure material, when heated slowly, would melt at 213°, then solidify to rhombic hexagonal plates and remelt at 223°. The mother liquors yielded a small amount of crystals, m.p. 231–233°; $\lambda\lambda_{max}$: 3.01, 5.70^{s}, 5.95^{s}, 6.06^{vs}, 6.13^{s} (Nujol). The assignments for the amide carbonyl bands may be made tentatively as 6.06 μ belonging to the α- and the hydrogen-bonded γ- or δ-benzamido groups, and 5.95 μ belonging to the non-bonded γ- (or) δ-benzamido group.

Higher-Melting (Geometric?) Isomer III of II.—Concentration of the mother liquors from II yielded a fair amount of slowly growing crystals consisting of buttons and tiny, hard needles. The former proved to be tribenzoyl ester II. Manual separation of the needles, followed by recrystallization from a large volume of ethanol or from methyl cellosolve gave tiny colorless rhombs, m.p. 247°. The infrared spectrum of III differs from II in the OH–NH region (shoulder at 3.04, broad band at 3.12 μ) and by the absence of a second amide band (5.70^{m}; 6.03^{sh}; 6.09^{vs}).

Anal. Calcd. for $C_{27}H_{25}N_3O_5$: C, 68.73; H, 5.34; N, 8.91; OCH_3, 6.57. Found: C, 68.43; H, 5.39; N, 8.95; OCH_3, 6.59.

α,δ-Dibenzoyl-γ-keto-L-ornithine Methyl Ester.—The compound was obtained in 78% yield from the parent methyl 2,4,5-tribenzamido-Δ^{4}-L-pentoate (II) as rosettes from ethanol–water, m.p. 168–170°; analytical sample, m.p. 170.5–171.5° (reported 171°[12,13] and 158°[14]).

Anal. Calcd. for $C_{20}H_{20}N_2O_5$: C, 65.20; H, 5.47; N, 7.61; OMe, 8.42. Found: C, 64.94; H, 5.35; N, 7.49; OMe, 8.52.

Infrared Spectrum.—A Nujol mull of this compound in one instance gave a complete separation of three carbonyl bands at 5.73^{s}, 5.78^{s}, 6.11^{vs}; other preparations gave only one single band in the ester region: 5.77 (Nujol); 5.73 ($CHCl_3$). Other bands: 2.91 ($CHCl_3$); 3.01 (Nujol); 3.03 (Nujol mull of product showing 3 carbonyls).

γ-Keto-L-ornithuric Acid (α,δ-Dibenzoyl-γ-keto-L-ornithine).—A mixture of 875 mg. of α,δ-dibenzoyl-γ-keto-L-ornithine methyl ester (IV, 28 ml. of 0.1 *N* sodium hydroxide and 2 ml. of methanol was heated for 40 minutes on the steam-bath. The slightly yellow solution was cooled and acidified with 0.2 ml. of glacial acetic acid, followed by 7 drops of concd. hydrochloric acid. The cloudy solution was extracted with two 20-ml. portions of chloroform and the extracts were dried over sodium sulfate and evaporated. The powdery residue was dissolved in 1 ml. of methanol, from which it crystallized nicely in almost quantitative yield upon the addition of 2 ml. of ether; after recrystallization from ethyl acetate, 98–102°. Recrystallization from methanol–ether gave the analytical sample, m.p. 116–118°. The compound was insoluble in chloroform or water, but dissolved readily in saturated sodium bicarbonate solution; $\lambda\lambda_{max}$: 3.06, 5.78^{vs}, 6.05^{s}, 6.50^{vs} μ (Nujol).

Anal. Calcd. for $C_{19}H_{18}N_2O_5 \cdot {}^1/_2CH_3OH$; C, 63.30; H, 5.45; N, 7.56. Found: C, 63.75; H, 5.79; N, 7.52.

Re-esterification.—Dibenzoylketo-L-ornithine (95 mg.) was dissolved in 3 ml. of methanol and diluted to 15 ml. with ether. A standardized solution of diazomethane in ether was added (1.4 ml. or 11.9 mg. CH_2N_2; 11.2 mg. required). Evaporation produced a white powder which after recrystallization from methanol–water and then methanol showed m.p. 168–171°. A mixed melting point with the authentic ester IV, m.p. 171–172°, was 167–172°. The infrared spectrum was identical with that of IV.

γ-Keto-L-ornithine Dihydrochloride.—A mixture of 4.0 g. of dibenzoyl-keto-L-ornithine ester, 18 ml. of water and 22 ml. of concd. hydrochloric acid was refluxed for seven hours and then chilled overnight. After filtration the residue was washed with 25 ml. of water and the combined filtrates (charcoal) were evaporated to dryness *in vacuo* at room temperature. The residue was taken up in 10 ml. of water and 10 ml. of ether. The aqueous portion was decolorized with charcoal in the cold, filtered and dried *in vacuo* at room temperature. The residue was a white gum with spots of darkening. The yield was 2.0 g. (85%). The gum decomposed rapidly in air, becoming black, and had to be used as prepared; λ_{max} 5.76, 6.28 μ (Nujol).

α-N-Carbamyl-γ-keto-L-ornithine.—Two grams of γ-keto-L-ornithine dihydrochloride, as a white gum, was dissolved in about 40 ml. of water, chilled in ice, and slowly mixed with 740 mg. of potassium cyanate. The solution of keto-ornithine was kept stoppered and the cyanate was added in small spatula quantities every 15 minutes for six hours. Half an hour after the addition was complete, precipitation commenced. After another half hour of chilling the white precipitate was removed by filtration (yield 369 mg., 21%). The product showed increasing decomposition, finally charring from 250–295° and was insoluble in ethanol, ether, chloroform, benzene and acetone, slightly soluble in methanol and soluble in water, from which it was obtained after three recrystallizations as cottony needles giving a red ninhydrin reaction.

Anal. Calcd. for $C_6H_{11}N_3O_4$: C, 38.09; H, 5.86; N, 22.21. Found: C, 37.92; H, 6.08; N, 21.18.

The mother liquor darkened considerably during filtration. Left in the cold overnight, it gave a second, almost colorless, crop. When the *p*H of the mother liquor was raised to 9 with sodium carbonate, ammonia was evolved.

Fischer Esterification of γ-Keto-L-ornithine.—A solution of one gram of γ-keto-L-ornithine in absolute methanol was saturated with hydrogen chloride at room temperature and kept under nitrogen for three days. The solution had darkened appreciably. When ether was added an oil separated. The solvent was decanted and the oil was taken up in methanol, mixed with an equal volume of benzene and concentrated. No crystallization occurred or could be induced so the solution was saturated with dry hydrogen chloride. On standing this deposited a bed of colorless, very hygroscopic needles. They were recrystallized by slow evaporation of a methanol solution as colorless needles, insoluble in acetone, melting to a clear froth from 103–110°; λ_{max} 5.74 μ (Nujol).

Anal. Calcd. for $C_5H_{10}N_2O_2 \cdot 2HCl$: C, 29.57; H, 5.96; N, 13.80. Found: C, 29.96; H, 6.05; N, 13.72.

According to the analytical figures this compound is not the methyl ester of γ-ketoörnithine; it could be the dihydrochloride of the free unacylated lactol (*cf.* formula iii in footnote 15).

Reduction of α,δ-Dibenzoyl-γ-keto-L-ornithine Methyl Ester (IV). A. Attempted Stepwise Reduction.—When the dibenzoyl ketoester IV was stirred with platinum black in acetic acid under hydrogen until *one mole of hydrogen* was absorbed, evaporation of the solution *in vacuo* and recrystallization from methanol gave a 25% yield of pure starting material and other fractions melting as low as 140–155°. When *two moles of hydrogen* was allowed to be taken up there was no sign of a change in the rate of absorption at either one or two moles, and starting material was recovered, though in low yield. No reduction occurred using 5%

(27) All melting points are corrected (Kofler block), all boiling points are uncorrected. The analyses were carried out by Dr. W. C. Alford and associates, Analytical Service Laboratories, National Institutes of Health.

palladized charcoal in methanol or using Adams catalyst in acetic anhydride–hydrobromic acid solution.

B. Reduction with Adams Catalyst in Ethanol.—The reduction of 5 g. of IV in 50 ml. of ethanol led to the uptake of somewhat more than 5 moles of hydrogen and stopped; the yield of reduction product was only 66%. The product precipitated readily from the ethanol during the reduction. Fractionation from methanol gave two fractions: a higher melting material which started to melt at 228°, then partially solidified and melted from 232–235°, $[\alpha]^{20}D$ −19.8° (20.383 mg. in 2 ml. of pyridine). The other fraction melted at 222–224°, $[\alpha]^{20}D$ −24.7° (19.110 mg. in 2 ml. of pyridine).

C. Complete Reduction of the Ketoester in Acetic Acid.—Ten grams of the ester IV in 250 ml. of glacial acetic acid was hydrogenated in the presence of 1 g. of Adams catalyst at 1900 pounds pressure. Filtration and concentration *in vacuo* gave an oil which crystallized rapidly when diluted with methanol. Recrystallization gave some material melting at 223–224°, but most melted over the range 219–235°. From three runs (32 g.) 11.7 g., or 37%, was recovered in the 223–225° range in the first crystallization.

α,δ-Dihexahydrobenzoyl-γ-hydroxy-L-ornithine Lactone, m.p. 242–243° (Presumably VIII).—Close examination of the melting point showed microcrystalline material in the melt. One crystallization of the 223–225° fraction from methanol gave 5.7 g. melting from 226–235°, and after two more crystallizations 1.2 g. of material was obtained melting sharply at 240–241°. Repeated and prolonged fractional crystallization of various fractions finally gave a total yield of 3.5 g. of colorless needles, m.p. 242–243°, $[\alpha]^{20}D$ −32.1° (*c* 2.0 in pyridine); $[\alpha]^{20}D$ −85 ± 2° (*c* 1.8 in 80% concd. hydrochloric acid and 20% dioxane).

Anal. Calcd. for $C_{19}H_{30}N_2O_4$: C, 65.11; H, 8.63; N, 7.99. Found: C, 65.31; H, 8.83; N, 7.99.

α,δ-Dihexahydrobenzoyl-γ-hydroxy-L-ornithine Lactone, m.p. 262.5–263° (Presumably IX).—Careful fractionation of the methanolic mother liquors from lactone, m.p. 243°, finally yielded about an equal quantity (3.4 g.) of very fine colorless needles, m.p. 262.5–263°; $[\alpha]^{20}D$ −4.27° (*c* 2.1 in pyridine).

Anal. Calcd. for $C_{19}H_{30}N_2O_4$: C, 65.11; H, 8.63; N, 7.99. Found: C, 65.11; H, 8.65; N, 7.91.

Mutarotation in Strong Acid.—A 2% solution of the higher melting lactone in a mixture of 80% concd. hydrochloric acid and 20% dioxane showed

Hours	$[\alpha]^{20}D$
0	− 3.9°
3	+23°
5	+20°
21.5	+20.4°

The material recovered after mutarotation had the same melting point and infrared spectrum as the starting material.

When 10 mg. each of the two lactones, m.p. 242 and 263°, were heated together in 2.5 ml. of methanol and the solution taken to dryness, the mixture showed m.p. 226–230° (softening and sublimation at 223–225°) and $[\alpha]^{20}D$ −18.1° (pyridine). The properties of the mixture obtained directly on hydrogenation (m.p. 222–223.5°, $[\alpha]^{20}D$ −24.7°) or from mother liquors of the pure lactones (m.p. 222–223°, $[\alpha]^{20}D$ −18.5°) are very similar, if not identical.

D. Hydrogenation with Raney Nickel in Methanol.—Twenty grams of pure α,δ-dibenzoyl-γ-keto-L-ornithine methyl ester (IV, m.p. 169–170°) dissolved in 350 ml. of methanol was hydrogenated in the presence of 5 g. of Raney nickel at a pressure of 1000 lb./sq. in. and a temperature of 120°. After five hours the pressure was constant. The catalyst was removed by filtration and the warm solution and washings (400 ml.) allowed to crystallize. Only a small crop (0.1 g.) of crystalline buttons was obtained at this point. The mother liquor was concentrated to one-fourth of its volume (100 ml.) and cooled very slowly in a bed of cotton. After two days a mixture of two distinctly different types of crystals was obtained which were separated mechanically under the microscope.

α,δ-Dihexahydrobenzoyl-γ-hydroxy-L-ornithine Lactone, m.p. 242–243° (Presumably VIII).—This isomer formed feathers of very fine long colorless needles, subliming from 225°, m.p. 234–236° (quiet colorless melt). After two recrystallizations from about 20 ml. of methanol 0.6 g. of fans of transparent needles, appearing under the microscope as long flattened needles or elongated rectangular platelets was obtained, m.p. 242–243°, undepressed on admixture with the analogous lactone obtained by hydrogenation with platinum. The infrared spectrum showed *three* characteristic carbonyl bands (5.69, 5.96, 6.06 μ).

Anal. Calcd. for $C_{19}H_{30}N_2O_4$: C, 65.11; H, 8.63; N, 7.99. Found: C, 65.36; H, 8.54; N, 7.81.

α,δ-Dihexahydrobenzoyl-γ-hydroxyornithine Lactone, m.p. 262° (Presumably IX).—The other crystalline modification formed spherical clusters of short, very fine needles, subliming to somewhat larger needles at 235°, melting at 253–255°. After two slow recrystallizations from about 25 ml. of methanol 0.25 g. of felted fine needles was obtained, m.p. 261–263°, undepressed on admixture with the high melting lactone obtained on catalytic hydrogenation with platinum at room temperature. The infrared spectrum showed only *two* bands in the carbonyl region (5.65 and 6.07 μ).

Anal. Calcd. for $C_{19}H_{30}N_2O_4$: C, 65.11; H, 8.63; N, 7.99. Found: C, 64.87; H, 8.48; N, 7.92.

The mother liquor on further concentration (70 ml.) deposited a mixture of crystals (3 g.) not separable mechanically. By fractionation with hot benzene more (~0.8 g.) of the low melting dihexahydrobenzoyl compound was obtained. The remainder of this 3-g. fraction consisted largely of the high melting dihexahydrobenzoyl isomer which was difficult to free from a compound lower in hydrogen, which was more soluble in ethyl acetate.

In order to obtain this compound, which apparently had to be looked for in the final methanolic mother liquors, the mother liquors were further concentrated to about 50 ml. and left in the cold room for two weeks. The crystalline deposit (14.1 g.) melted unsharply at 200–225° and showed only two peaks in the carbonyl region.

The final mother liquor was evaporated to dryness. There was obtained 6.5 g. of colorless powder, largely soluble in chloroform in which the following bands were shown: 2.92, 5.63^{m}, 5.76^{s}, 6.00^{vs}, 6.06^{vs}, 6.22^{m} μ. This fraction was dissolved in 250 ml. of hot benzene and poured onto 300 g. of alumina (Woelm, neutral, activity I). Nothing was eluted with benzene or ether. With ethyl acetate containing 4% acetone (each time 250 ml.) five fractions (I–V) were obtained.

Compound $C_{19}H_{18}N_2O_4 \cdot {}^1/_2H_2O$ (Presumably α,δ-Dibenzoyl-γ-hydroxyornithine Lactone, V).—Fraction I consisted of 0.25 g. of a crystalline powder, soluble in methylene chloride, showing only two carbonyl bands (*cis* series) in Nujol: 5.58^{s}, 6.10^{vs} and a weak phenyl band at 6.22^{w} μ. It was sparingly soluble in ethyl acetate, and appeared on slow cooling in fine needles, m.p. 234°.

Anal. Calcd. for $C_{19}H_{18}N_2O_4 \cdot {}^1/_2H_2O$: C, 65.70; H, 5.51; N, 8.07. Found: C, 65.37; H, 5.98; N, 8.01.

α,δ(or δ,α)-Benzoyl-hexahydrobenzoyl-γ-hydroxyornithine Lactone (VI).—Fraction II (0.45 g.) was recrystallized from ethyl acetate in which it is not very soluble. After slow recrystallization (four days at 30°) cushions of short stout colorless needles were obtained, m.p. 207–210° (sintering at 187°). Further recrystallizations from a little methanol separated this fraction into two components of which the one less soluble in methanol formed long lissome needles, m.p. 218–222°, $[\alpha]^{20}D$ +14.6° (pyridine, *c* 1.0).

Anal. Calcd. for $C_{19}H_{24}N_2O_4 \cdot {}^1/_4H_2O$: C, 65.42; H, 7.08; N, 7.97. Found: C, 65.03; H, 7.00; N, 7.92.

TABLE III

PRODUCTS AND YIELDS IN THE REDUCTION OF α,δ-DIBENZOYL-γ-KETO-L-ORNITHINE METHYL ESTER (IV) WITH RANEY NICKEL AT 120°

γ-Hydroxy-L-ornithine lactone derivative		M.p., °C.	Approx. yields, %
Low melting dihexahydrobenzoyl (VIII)		243	~80
High-melting dihexahydrobenzoyl (IX)		263	~10
(*cis?*)-α-Benzoyl-δ-hexahydrobenzoyl	(VI)	192–194	~5
(*cis?*)-α-Hexahydrobenzoyl-δ-benzoyl	(VI)	218–222	
(*cis?*)-α,δ-Dibenzoyl (V)		234	~2

TABLE IV

SEPARATION AND PROPERTIES OF THE TWO DIASTEREOISOMERIC DIHEXAHYDROBENZOYL-γ-HYDROXYORNITHINE LACTONES (VIII AND IX)

	Pure low melting diastereoisomer (presumably VIII)	Pure high melting diastereoisomer (presumably IX)	Mixture (VII) initially present, or obtained from mother liquors after separation of VII and IX
M.p., °C.	242–243	262.5–263	222–223
$[\alpha]^{20}D$ in pyridine	−32.1°	−4.27°	−18.5°
$[\alpha]^{20}D$ in 80% dioxane + 20% concd. HCl	−85° ± 2°[a] constant	−3.9° (t = 0)[a] +20.4° (t = 2 hr.)	−33.5[b] No change after 2 hr.

[a] The recovered material had the same m.p. and mixed m.p. and the same infrared spectrum as the starting material. [Calculated for $[\alpha]D$ of equimolar mixture of VIII and IX: −85° + 20.4 = 64.6:2 = 32.3°].

TABLE V

PROPERTIES OF VARIOUS MIXTURES OF DIASTEREOISOMERIC γ-HYDROXYORNITHINES AS THE DI- AND MONOHYDROCHLORIDES AS WELL AS THE FREE LACTONES OR OPEN ACIDS AND THEIR SODIUM SALTS

Source, m.p., composition	Dihydrochloride $[\alpha]D$ Initial	Final	Monohydrochloride (1 m. of NaOH) $[\alpha]D$ Initial	Final	Free lactone or open acid[a] (2 m. of NaOH) $[\alpha]D$ Initial	Final	With excess base (3 m. of NaOH)[b] $[\alpha]D$ Initial	Final	+ Excess acid
From original mixture VII, m.p. 200–201° (mixture of 4 isomers)	−17.5° (in water) −13.5° (in 0.1 N HCl)	+13.7° +13.7°[c]	+8.2° (constant)		+3.3°	−1.1° (23 hr.)	−2.3°	−1.8° (3 days)	Not measured
From pure VIII m.p. 220° 72% Ls-*erythro* and 28% Ds-*threo*	−53.8°	+17.1°	+16.3° (45 min.)	+4.8° (43 hr.)		+13.6° (22 hr.)	+5.26° in buffer	... *p*H 7.8	+12.30° (constant)
From pure IX m.p. 217–223° 69% Ls-*threo* and 31% Ds-*erythro*	+7.02°	+22.3° (30 hr.)	0° (constant)		+3.72°	+10.12°	2.48° in buffer +7.69°	*p*H 7.8[d] −8.79°	+5.6° (constant)

[a] Figures calculated for free acid. [b] Figures calculated for sodium salt. [c] Picrate obtained from dihydrochloride after mutarotation differs from that of starting material. Based on the initial reading the calculated ratio would be about 51% of VIII and 49% of IX. [d] These values were observed when the dihydrochlorides were dissolved in 1/10 M phosphate buffer *p*H 7.8.

Two further recrystallizations raised the m.p. to 226–229°.

Anal. Calcd. for $C_{19}H_{24}N_2O_4$: C, 66.26; H, 7.02; N, 8.13. Found: C, 66.13; H, 6.83; N, 8.06.

The more methanol-soluble part obtained from mother liquors appeared in fine needles, m.p. 192–194°, $[\alpha]^{20}D$ +4.98° (pyridine, c 1.0).

Anal. Calcd. for $C_{19}H_{24}N_2O_4$: C, 66.26; H, 7.02; N, 8.13. Found: C, 66.81; H, 6.78; N, 8.24.

The subsequent fractions (III–V) contained high-melting dihexahydrobenzoyl lactone contaminated with monobenzoyl lactone. The material eluted from the column using pure acetone and subsequently chloroform and methanol amounted to 2.9 g.

γ-Hydroxy-L-ornithine Lactone Dihydrochloride (Preponderantly X). A. From the Hydrolysis of Lactone, m.p. 243°.—One-half gram of the dihexahydrobenzoyl lactone, m.p. 240–242°, was refluxed in a mixture of 10 ml. of concd. hydrochloric acid and 10 ml. of water for 2.5 hours. The clear solution was extracted with four 10-ml. portions of pure ether. Titration of the ether showed 1.95 equivalents of hexahydrobenzoic acid. When the aqueous portion was dried *in vacuo* in the desiccator, it crystallized. Two triturations with 2 ml. of absolute ethanol, followed by ether, gave a white, non-hygroscopic powder which began to brown at 208°, softened at 218° and decomposed at 220°.

Anal. Calcd. for $C_5H_{10}N_2O_2 \cdot 2HCl$: C, 29.57; H, 5.96; N, 13.80. Found: C, 29.66; H, 5.87; N, 13.86.

Mutarotations (Table V).—A solution of 20.766 mg. of the dihydrochloride in 1 ml. of 0.1 M phosphate buffer of *p*H 7.8 gave the following values, using the equation[28]

$$k_1 + k_2 \frac{1}{t} \log \frac{r_0 - r_\infty}{r - r_\infty}$$

The average change of rotation during the first ten minutes was −0.74°/min.; t_0 was the first reading taken about one minute after the preparation of the solution. The calculated values for pure L-*erythro*-hydroxyornithine, assuming only epimerization at C(2), would be $[\alpha]^{20}D$ −48.4° → +7.2° (in buffer *p*H 7.8) and −85.8° → +44° (in water).

t (hours)	$[\alpha]^{23}D$	$r - r_\infty$	$\frac{r_0 - r_\infty}{r - r_\infty}$	$k_1 + k_2$	
0	−31.9	39.72	....		
0.5	−13.40	21.22	1.870	0.54	av. 0.34
1	− 7.14	14.96	2.68	.43	
2	+ 1.06	7.76	5.12	.36	
3	+ 2.99	4.83	8.22	.30	
3.5	+ 3.67	4.15	9.57	.28	
4	+ 4.15	3.67	10.83	.26	
5	+ 4.87	2.95	13.46	.22	

When a mutarotated aqueous solution giving values $[\alpha]D$ −51.5° → 12.0° was evaporated to dryness (at 20° *in vacuo*) with excess 2 N hydrochloric acid, the new initial rotation in water was $[\alpha]D$ −34.6°, indicating incomplete reclosure of the lactone or epimerization (alkyl–oxygen fission?) or partial polymerization in the course of lactone opening.

Picrate.—Addition of saturated aqueous picric acid to a water solution of the dihydrochloride precipitated *yellow rosettes* which browned at 215–218° and decomposed at 229–231°. The picrate was insoluble in cold alcohol, only slightly soluble in acetone. In recrystallization from water mutarotation apparently occurred (see below) and yellow needles were obtained which turned dark brown at 238° and decomposed with melting from 244–248°.

Anal. Calcd. for $C_5H_{10}N_2O_2 \cdot 2C_6H_3N_3O_7$: C, 34.70; H, 2.74. Found: C, 35.23; H, 3.11.

The same picrate was obtained when a solution of 25 mg. of the lactone in 1 ml. of water was left to mutarotate for 48 hours and then mixed with 1 ml. of saturated picric acid. Crystallization started within 10 minutes giving irregular yellow plates which turned black at 243° with distillation,

(28) *Cf.* F. J. Bates, "Polarimetry, Saccharimetry and the Sugars," Circular of the National Bureau of Standards, C440, U. S. Government Printing Office, Washington, 1942, p. 442.

paralleling the behavior of the picrate from lactone before mutarotation after it had been recrystallized.

Reineckate.—The addition of free aqueous Reinecke acid, prepared by ether extraction of a solution of Reinecke salt in 2 *N* sulfuric acid, caused the immediate precipitation of raspberry-colored crystals, easily soluble in acetone but for a small residue; $[\alpha]^{20}_{6234}$ −11.4° (acetone, *c* 0.7).

Assay by Stein and Moore Technique.—The method of Hirs, Moore and Stein[29] as modified by Piez[17] was utilized to assay the purity and composition of the lactone. An aliquot containing approximately 1/2 mg. of lactone dihydrochloride in 0.1 *M* phosphate buffer of *p*H 7.8 (less basic buffer solutions failed to achieve satisfactory elution and resolution) was left at 20° for 48 hours to effect mutarotation and then passed through a 100 × 0.9 cm. column of Dowex-50 (shorter columns failed to effect separation) surrounded by a jacket containing water thermostated at 37°. Slight pressure was applied to keep the flow rate at 2 drops/minute; 0.1 *M* phosphate buffer of *p*H 7.8 was used as effluent. One-ml. samples were collected with an automatic fraction collector and analyzed by the ninhydrin method[30] utilizing λ_{max} 570 mμ. The marker was "natural" hydroxylysine[18] containing 82% δ-hydroxy-L-lysine and 18% allohydroxy-D-lysine as obtainable by acid hydrolysis of gelatin. When collection was started at the time the sample and two 1-ml. washings had just gone into the column, the highest concentrations were found as follows: hydroxy-L-lysine, tubes 260–262; allohydroxy-D-lysine, tubes 277–280; *threo*-hydroxy-D(or L-)-ornithine, tubes 309–312; *erythro*-hydroxy-L(or D-)-ornithine, tubes 325–328. The hydrolysis of lactone, m.p. 242°, after 2.5 hours of refluxing in 6 *N* HCl gave a dihydrochloride containing 28% of the more elutable diastereoisomer (presumably D-*threo*-(or allo)-hydroxyornithine, XV) and 72% of the less elutable amino acid (presumably L-*erythro*-γ-hydroxyornithine, XVIII). When the time of hydrolysis was shortened to one hour or 45 minutes there was essentially only one peak (tubes 325–328) observed indicating L-*erythro*-γ-hydroxyornithine (XIX) of a purity of 90 ± 10%.

γ-Hydroxy-L-ornithine Lactone Dihydrochloride (Preponderantly XIII) B. From the Hydrolysis of Lactone, m.p. 263°.—One gram of lactone, m.p. 262°, was boiled with 20 ml. of water and 20 ml. of concd. hydrochloric acid for 7 hours, dissolving after 5 hours to yield a slightly amber solution. Extraction with four 20-ml. portions of ether and evaporation of the water layer gave a gum which, on standing for four days in 20 ml. of absolute ethanol, became a hygroscopic crystalline powder. Repeated trituration with ethanol and centrifugation gave a colorless powder which became soft and began to brown at 217°, melting with frothing at 223°. In subsequent runs the time of hydrolysis was shortened to three hours.

Anal. Calcd. for $C_5H_{10}N_2O_2 \cdot 2HCl$: C, 29.57; H, 5.96; N, 13.80. Found: C, 29.61; H, 6.05; N, 13.95.

Mutarotation.—A solution of 19.09 mg. of lactone dihydrochloride in 1 ml. of 0.1 *M* phosphate buffer of *p*H 7.8 gave the following readings at 20°

Hours	$[\alpha]^{20}D$	$r - r_\infty$	$\frac{r_0' - r_\infty}{r - r_\infty}$	$k_1 + k_2$	
0	+7.69	16.48	...	..	
1.5	+1.05	9.84	1.67	0.15	Av. 0.12
3.0	−2.93	5.86	2.81	.15	
4.5	−4.61	4.18	3.94	.13	
21.5	−8.28	0.51	32.3	.070	
24	−8.79				

The mutarotation coefficient,[28] $k_1 + k_2$, changes as the reaction proceeds. The reason for this is the presence of four different species (70% L-*threo*-hydroxyornithine as lactone and free acid, 30% D-*erythro*-hydroxyornithine as lactone and free acid) in the mutarotated solution. The calculated values for pure L-*threo*-hydroxyornithine lactone before and after mutarotation would be +10.61° → −9.5° (in buffer *p*H 7.8) and −28.4° → 52.1° (in water).

Comparative Mutarotations in Different Solvents.—11-95 mg. of the above lactone dihydrochloride in 0.5 ml. of 1/10 *N* sodium chloride solution gave a final value of $[\alpha]^{20}D$ +8.1 ± 0.1°. The same amount dissolved in 0.5 ml. of 1/10 *N* sodium hydroxide was left for 48 hours and the solution brought to *p*H 7, $[\alpha]^{20}D$ +6.48 ± 0.1°.

Manometric Observation of Lactone Opening by Reaction with Chloramine T.—Manometric determinations were run according to a modification of the method of P. P. Cohen.[31] Solutions of the amino acid (hydroxyornithine lactone dihydrochloride) were made up 0.003 to 0.004 *M*; 0.1 to 0.3 ml. of these solutions were placed in the side-arms of Warburg vessels. To each cell was added 1 ml. of 10% Chloramine T solution, 0.7 ml. of citrate buffer *p*H 4.7 and sufficient water to bring the total volume (including the solution in the side-arm) to 2 ml.

The solutions were shaken at 25° until equilibrium was established and the amino acid solution was added from the side-arm. There was a brisk evolution of CO_2 which usually became negligible after 15 minutes. The results are summarized in Table I.

Picrate.—When 170 mg. of the dihexahydrobenzoyl-γ-hydroxy-L-ornithine lactone, m.p. 262°, was refluxed with 10 ml. of 6 *N* hydrochloric acid the lactone dissolved to form a clear pale yellow solution at the end of 12 hours. The solution was allowed to cool, diluted with 10 ml. of water and extracted with three 10-ml. portions of ether. The ether contained 1.5 equivalents of cyclohexanecarboxylic acid. The aqueous solution was dried *in vacuo* at room temperature to a slightly yellow-brown viscous material which did not become readily crystalline on trituration with alcohol. To a solution of this material in 1 ml. of water was added 10 ml. of saturated aqueous picric acid. Needles (65 mg., no darkening below 240°, dec. above 250°) soon crystallized, insoluble in hot methanol or acetone, slightly water-soluble and readily soluble in, and recrystallizable from, warm water. The recrystallized picrate began to brown at about 220°, spotting and subliming at about 239°, becoming a mass of black rods at 249°.

Anal. Calcd. for $C_5H_{10}N_2O_2 \cdot 2C_6H_3N_3O_7$: C, 34.70; H, 2.74; N, 19.05. Found: C, 34.73; H, 2.87; N, 18.02, 18.07.

No weight loss at 100°.

Again, the mutarotated lactone dihydrochloride gave a picrate identical with the one from unmutarotated lactone *after* recrystallization from water.

Direineckate.—Free reinecke acid added to the aqueous solution of the dihydrochloride formed a pink crystalline precipitate, easily soluble in acetone but for a small light-pink, on washing almost colorless, residue amounting invariably to about 10% of the weight of the reineckate; m.p. 214–230° dec.; $[\alpha]^{20}_{6234}$ −8.3° (acetone, *c* 1.1).

Anal. Calcd. for $C_5H_{10}N_2O_2 \cdot 2H[Cr(NH_3)_2(SCN)_4]$: C, 18.97; H, 3.67; N, 23.83. Found: C, 19.22; H, 4.01; N, 24.01.

Assay by Dowex-50.—The analysis of the ornithine lactone dihydrochloride (after mutarotation in 0.1 *M* phosphate buffer of *p*H 7.8) by the method described above showed that the action of 6 *N* hydrochloric acid for 3–7 hours leads to mixtures containing 2/3 of the more easily eluted amino acid (presumably L-*threo*-hydroxyornithine) and 1/3 of the epimerized compound (presumably D-*erythro*-hydroxyornithine); for the above lactone the ratio was 68%:32%. The hydrolysis of 20 mg. of lactone, m.p. 263° in 2 ml. of 6 *N* hydrochloric acid for 45 minutes gave mainly one peak (309–312) and a very small second peak (325–328°) indicating 90% L-*threo*-hydroxyornithine. The margin of error in such disparate ratios is higher (∼±10%) than in a 70:30 ratio (∼±5%).

Incomplete hydrolysis is observed when the time of refluxing is shortened to 15 minutes. Of 20 mg. of lactone about 5 mg. of starting material (m.p. 260–263°) was undissolved at that time. The acid solution after ether extraction to remove some hexahydrobenzoic acid and evaporation left a residue which was insoluble in hot water (m.p. 245–250° dec.), 230° crystalline transformation from small prisms to birefringent fine needles.[32]

(29) C. H W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **195**, 669 (1952).

(30) S. Moore and W. H. Stein, *ibid.*, **176**, 367 (1948).

(31) P. P. Cohen, *ibid.*, **136**, 571 (1940).

(32) Incomplete hydrolysis needs to be further investigated to confirm that the expected monoacyl derivative is the α-hexahydrobenzoyl-γ-hydroxyornithine formed *via* acyl migration N → O and subsequent ester hydrolysis and not the δ-acyl compound which is observed in the acid hydrolysis of ornithuric acid [P. Karrer and M. Ehrenstein, *Helv. Chim. Acta*, **9**, 323 (1926)]; base hydrolysis of ornithuric acid leads to α-benzoylornithine [S. P. L. Sörensen, M. Höyrup and A. C. Andersen, *Z. physiol. Chem.*, **76**, 52 (1912)]. Lysuric acid forms ε-benzoyllysine on controlled hydrolysis with base or acid.

Hydrolysis for 20 hours did not significantly change the above 2:1 ratio. The analysis of identical samples after mutarotation in 0.1 *M* phosphate buffer *p*H 7.8 or after mutarotation in dilute alkali at *p*H 12 (to eliminate any possible alkyl–oxygen fission) did not show different ratios within the limit of experimental error.

C. Hydrolysis of the Lactone Mixture (VII), m.p. 222–224°.—One-half gram of lactone, m.p. 222–224°, from the mother liquors of the two pure lactones was refluxed with 20 ml. of 6 *N* hydrochloric acid yielding a clear solution in less than one hour. Gentle refluxing was continued for two more hours, after which it was worked up as the previous runs. One week's digestion in absolute alcohol was necessary to convert the dried amber gum to a crystalline white solid which was very hygroscopic. It softened at 206° and melted with frothing and discoloration at 210–215°.

Anal. Calcd. for $C_5H_{10}N_2O_2 \cdot 2HCl$: C, 29.57; H, 5.96; N, 13.80. Found: C, 29.86; H, 6.02; N, 14.10.

Mutarotation.—Solutions of the lactone dihydrochloride in 0.1 *N* hydrochloric acid showed

Time, hr.	0	3	21	43
$[\alpha]^{20}D$	−13.5°	−7.7°	9.7°	13.7°

The **dipicrate** formed pale yellow needles, m.p. 249–251° dec.

Anal. Calcd. for $C_5H_{10}N_2O_2 \cdot 2C_6H_3N_3O_7$: C, 34.70; H, 2.74; N, 19.05. Found: C, 34.97; H, 2.99; N, 18.82.

Free Lactone.—A solution of 508 mg. of lactone dihydrochloride in 10 ml. of water was stirred with a slurry of Dowex-50 acetate until the liquid phase no longer showed a test for chloride. The mixture was centrifuged and the aqueous phase was decanted. The Dowex-50 acetate was twice more stirred with 50-ml. portions of water, centrifuged, and the aqueous phase decanted. The combined solution was taken to dryness *in vacuo* at 30°, the residue taken up in methanol and, after filtration, again taken to dryness *in vacuo*. It was then triturated with 3–4 ml. of absolute alcohol and the procedure repeated. Finally the solid was washed with ether and centrifuged. The yield was essentially quantitative. The colorless solid liquefied at 50–55°. It was insoluble in hot or cold ethanol, but soluble in water or methanol. It could not be recrystallized from any combination of these, and formed a gel in methanol.

Anal. Calcd. for $C_5H_{10}ON_2O_2 \cdot H_2O$: C, 40.53; H, 8.11; N, 18.91. Found: C, 40.38; H, 7.54; N, 18.43.

The solid material turned to a viscous mass before the analysis could be completed.

Silver hydroxide could not be used for the liberation of the lactone from the dihydrochloride, since it was rapidly reduced to silver.

The Action of Nitrosyl Chloride on γ-Hydroxyornithine (Lactone).—The solution of nitrosyl chloride was prepared by the addition of 0.5 g. of silver nitrite to 6 ml. of 1 *N* hydrochloric acid at 0° and filtration from the silver chloride. This solution was added to a solution of 200 mg. of either lactone dihydrochloride (from various hydrolyses of dihexahydrobenzoyl lactones, m.p. 243 and 263°) in 10 ml. of ice-cold 9 *N* hydrochloric acid. Freshly prepared solutions of the lactone dihydrochlorides reacted with vigorous bubbling and with immediate discharge of the yellow color of the nitrosyl chloride solution. Solutions of open γ-hydroxyornithine which had been allowed to mutarotate for several days in a small volume of water, showed much slower reaction with nitrosyl chloride at comparable acid concentration. The reaction mixture was allowed to stand for ten minutes in the cold and then heated to 55° for 30 minutes (for variations of these conditions see Table II) and then dried *in vacuo*. Whereas various hydrolysates from lactone, m.p. 263°, gave colorless solutions on warming with NOCl, most reaction mixtures of NOCl with the lactone from hydrolysis of the dihexahydrobenzoyl lactone, m.p. 243°, gave red solutions.

The residue was analyzed for total nitrogen by the micro method according to Kjeldahl–Nessler and for the liberation of CO_2 with Chloramine T at 25° in a Warburg manometer.[31] The following values were obtained: 9.83% total nitrogen and 11.5% CO_2 for the product from lactone before mutarotation, and 9.3% N_2 and 7.6% CO_2 for the product from mutarotated γ-hydroxyornithine (in each case obtained by 3-hour hydrolysis in 6 *N* hydrochloric acid from the lactone, m.p. 263°).

Calculation of the Results.—The following table summarizes the results which indicate a preferential reactivity of the α-amino group

Reaction products	Symbol	Mol. wt.	Theory, % N_2	Theory, % CO_2	Composition of the reaction mixture, % From lactone before mutarot.	Composition of the reaction mixture, % From hydroxyamino acid after mutarot.
γ-Hydroxyornithine (lactone)·2HCl	W	203.2	13.8	21.7	37	29
α-Chloroacid·HCl	X	186.2	7.5	..	53	65
δ-Chloroacid·HCl	Y	186.2	7.5	23.6	10	6
α-δ-Dichloro-γ-valerolactone	Z	169.2	0	0	0	0

The calculations are based on the assumption that with one mole of nitrosyl chloride the amount of α,δ-dichloroacid is zero or negligible. The figures for W, X and Y in the above table are obtained from the following equations (a) before mutarotations: 13.8W + 7.5X + 7.5Y = 9.83; 21.7W + 23.6Y = 11.5; W + X + Y = 1; (b) after mutarotation: 13.8W + 7.5X + 7.5Y = 9.3; 21.7W + 23.6Y = 7.6; W + X + Y = 1.

With excess nitrosyl chloride, assuming that little or no δ-chloroacid is formed, the amount of 2-chloroacid rises to approximately 70% before, and 80% after, mutarotation.

Cyclization of the Mixture of α-(or δ)-Chloro-δ-(or α)-amino-γ-hydroxy-valeric Acids to a Mixture of (Allo)Hydroxyprolines.—The dry product from the reaction with nitrosyl chloride was taken up in 2 ml. of water and then heated with 10 ml. of 0.2 *N* barium hydroxide at 100° for ten minutes (for variations of these conditions see Table II). The solution was neutralized and freed from barium ions by the addition of sulfuric acid. The filtrate from the barium sulfate was taken to dryness and analyzed for natural and allohydroxyproline.

Separation of Natural and Allohydroxyproline on Dowex-50.—An aliquot containing approximately 1 mg. of hydroxyproline in citrate buffer *p*H 3.3 was passed through a 50 × 0.9 cm. column of Dowex-50 maintained at 37°, under sufficient pressure to pass about 2 drops per minute. Citrate buffer of *p*H 3.3 was used as effluent. An automatic fraction collector collected 1-ml. samples. These samples were analyzed by the ninhydrin method of Moore and Stein[29,30] observing absorbancy at 440 mμ. The column was first standardized with *normal* and *allo*hydroxyproline for determination of the absorbancy factors. The collection was started when the sample had just gone into the column, the maximum concentration of *normal* hydroxyproline was found in the 37th tube, and of the *allo* isomer in the 47th. A marker such as methionine sulfoxide (29th tube) was always used. The ratios of *normal* to *allo*hydroxyproline for various cyclization mixtures are recorded in Table IV (see also Fig. 5).

Characterization and Partial Separation of *Normal* and *Allo*hydroxyproline as Reineckates. A. Hydroxy-L-proline Reineckate.—Purest hydroxy-L-proline (0.5 g., $[\alpha]^{22}D$ −75.2°) in 5 ml. of water was treated with a slight excess of a freshly prepared 10% aqueous solution of Reinecke acid, made from Reinecke salt by suspending it in 2 *N* sulfuric acid and extracting with ether. The reineckate crystallized slowly in pink platelets which were washed with water; m.p. 152–153° (red-violet viscous melt, no decomposition). The compound is very acetone-soluble, 50 mg. dissolving in as little as 0.5 ml. of acetone; $[\alpha]^{20}_{6234}$ −38.9° (acetone, *c* 2.0).

Anal. Calcd. for $C_5H_9NO_3 \cdot H[Cr(NH_3)_2(SCN)_4]$: C, 24.01; H, 3.58; N, 21.79. Found: C, 23.95; H, 3.81; N, 21.68.[33]

B. Di-[allohydroxy-D-proline] Reineckate.—In contrast to the slowly crystallizing reineckate of normal hydroxyproline that of allohydroxyproline formed immediately in water as mush of pink crystals which were much less acetone-soluble than the epimeric reineckate; pink-violet cubes,

(33) A compound prepared with Reinecke *salt* has previoulsy been reported as having m.p. 248° and the composition $C_5H_9NO_3 \cdot H[Cr(NH_3)_2(SCN)_4] \cdot NH_4[Cr(NH_3)_2(SCN)_4].3H_2O$; J. Kapfhammer and R. Eck, *Z. physiol. Chem.*, **170**, 294 (1927).

c :rkening at 165°, m.p. 177–180° (viscous melt). A sample which was washed with acetone several times instead of with water, gave similar analytical figures (C, 29.92; H, 4.66) but did not melt up to 270°; $[\alpha]^{20}_{6234}$ +44.4° (acetone, *c* 0.56).

Anal. Calcd. for $2\cdot C_5H_9NO_3\cdot H[Cr(NH_3)_2(SCN)_4]$: C, 28.91; H, 4.33; N, 19.27. Found: C, 29.26; H, 4.28; N, 19.58.

Partial Separation of Hydroxy-L-proline from Allohydroxy-D-proline by Fractional Precipitation of the Reineckates.—To a solution of 0.2 g. of hydroxy-L-proline and of 0.2 g. of allohydroxy-D-proline in 3 ml. of water was added 10 drops of a 10% aqueous solution of Reinecke acid. The pink precipitate which formed immediately was washed 3 times with 3 ml. of water and then with 2 ml. of acetone. The rotation of this reineckate (150 mg.) $[\alpha]^{20}_{6234}$ +36.0° (acetone, *c* 0.46) shows that allohydroxyproline of about 90% purity is precipitated first by reinecke acid. Of subsequent fractions formed in the presence of excess reinecke acid those that formed slowly on standing in the cold room showed decreasing rotations ($[\alpha]^{20}_{6234}$ +12.6°) which were still positive. The isolation of the more soluble hydroxy-L-proline reineckate from the last mother liquors was not attempted. Chromatographic separation of the acetone solution of a mixture (1:1) of the two reineckates on a column of neutral aluminum oxide did not lead to the formation of discrete bands or zones.

Stereochemical Correlation of the Hydroxyl Group of γ-Hydroxyornithine Lactones (X–XIII) by Transformation to a Mixture of Natural and Allohydroxyproline. A. Chromatographic and Polarimetric Analysis of Hydroxyprolines from Hydrolysate of Dihexahydrobenzyl Lactone, m.p. 243°.—A freshly prepared solution of 400 mg. of lactone dihydrochloride from hydrolysis (three hours) of dihexahydrobenzoyl lactone, m.p. 243°, was immediately treated with 8 ml. of the nitrosyl chloride solution as described above. The reaction slowed down after the addition of 4 ml. and the solution turned red when heated at 55° for 18 minutes. The reaction mixture was dried *in vacuo*, dissolved in 2 ml. of water, heated with 10 ml. of 0.2 *N* barium hydroxide at 100° for 10 minutes, neutralized with 1 *N* sulfuric acid and dried *in vacuo*.

The dried residue was dissolved in 3 ml. of water and treated with 0.6 ml. of a 20% reinecke acid solution. The most insoluble reineckate was separated immediately. According to the high nitrogen analysis (found: N, 22.8) this material was mainly the very insoluble direineckate of unchanged starting material (calcd.: N, 23.8). An excess of reinecke acid was added and the solution left in the cold room overnight. A precipitate of 185 mg. of raspberry crystals was collected, $[\alpha]^{20}_{6234}$ −15.3°[34]; 150 mg. of this reineckate dissolved easily in acetone. It was converted to the sulfate by the addition of excess 2 *N* sulfuric acid and extraction of the reinecke acid with ether. The colorless solution was then freed of sulfate ions with barium hydroxide. The dried hygroscopic lacquer of the free amino acid weighed 22.2 mg. Rotation of a sample dried over phosphorus pentoxide was $[\alpha]^{20}_{D}$ −20.0°.

Regardless of mechanism only one of the two possible pairs of hydroxyprolines is to be expected: (a) hydroxy-D-proline (+75.2°) + allohydroxy-L-proline (−58.1°), or (b) hydroxy-L-proline (−75.2°) + allohydroxy-D-proline (+58.1°). In order to obtain the observed rotation, the theoretical ratios must be: (a) hydroxy-D-proline 29% + allohydroxy-L-proline 72% or (b) hydroxy-L-proline 59% + allohydroxy-D-proline 41%. Ion-exchange analysis of the isolated amino acid mixture gave a ratio, normal to allo, of 69:31,[35] thus showing the mixture to be normal hydroxy-L-proline plus allohydroxy-D-proline; the stereochemistry of the hydroxyl, therefore, is of L_G configuration.

B. Analysis of Hydroxyprolines from Hydrolysate of Dihexahydrobenzoyl-γ-hydroxy-L-ornithine Lactone, m.p. 263°.—When 400 mg. of lactone dihydrochloride from 3-hour hydrolysis of lactone, m.p. 263°, was treated with nitrosyl chloride as described under A, the fractional precipitation of the reineckates (total yield 330 mg.) gave fractions averaging a rotation of $[\alpha]^{20}_{6234}$ −6.5°; a later fraction of 134 mg. was converted into the free amino acid as described above yielding 23.5 mg. of crystalline amino acid, $[\alpha]^{20}_{D}$ −21.3°. To give the rotation found, the theoretical ratios must be: (a) hydroxy-D-proline 28%; allohydroxy-L-proline 72%; (b) hydroxy-L-proline 60%; allohydroxy-D-proline 40%. Ion-exchange analysis showed a ratio of normal to allo of 39:61, showing that the mixture is one of normal hydroxy-D-proline plus allohydroxy-L-proline and the hydroxyl group is of D_G configuration.

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(34) The advantage of fractionation with reinecke acid of the ternary mixture of hydroxyornithine and the two hydroxyprolines is a twofold one: (a) separation of the amino acids from other ions, (b) separation from starting material which is precipitated as the direineckate before the diallohydroxyproline reineckate. No significant separation of the two hydroxyprolines was achieved nor desired; thus the composition of the second reineckate fraction was representative of the ratio of hydroxyprolines in the entire fraction, as independent analyses of numerous cyclization mixtures obtained in yields of 20–60% (Table II) demonstrate.

(35) Ion-exchange analysis showed that only 47% of the amino acid isolated was hydroxyproline. The low recovery of hydroxyproline suggests that the conversion to chloraminohydroxy acid was incomplete and that unchanged hydroxyornithine was present in the insoluble reineckate fraction. If one assumes that 52% of the starting material did not react, then during the subsequent stages it would have mutarotated to −6.0. Calculated for 32.5% of normal hydroxy-L-proline ($[\alpha]^{20}_{D}$ −75.2°): −24.5°; 14.8% of allohydroxy-D-proline ($[\alpha]^{20}_{D}$ +58.1°): +8.6°; 52.7% of mutarotated hydroxyornithine lactone ($[\alpha]_{D}$ − 6.0°): −3.2°. Resultant rotation of the amino acid residue was −19.1°; found −20.0°.