The data in columns 6 and 7 of Table III compare rates of ultraviolet decomposition of the purines at high intensity with adenine under oxygen and at a lower intensity with guanine under nitrogen. Here the inhibiting effect of the amino group is apparent from the relative decomposition rates of adenine *vs.* hypoxanthine and of guanine *vs.* xanthine under both oxygen and nitrogen. The inversion of k_{O_2}/k_{N_2} which occurs at guanine is shown in column 5 of the table.

Adenine (7.8×10^{-5} *M*) is essentially completely destroyed at the end of 24 hours irradiation under aerobic conditions, whereas under N_2, more than 90% remains at the end of this period. This suggests oxidative breakdown with the amino group a likely point of attack

$$RNH_2 \xrightarrow[h\nu]{[O]} [R{\cdot}NH{:}OH] \xrightarrow[h\nu]{H_2O} ROH$$

The isolation of hypoxanthine in the photochemical oxidation of adenine is further evidence in favor of this step.[11]

The following sequence of steps in the photochemical decomposition of adenine is suggested by the isolation of hypoxanthine—namely, (I) → (II) → (IV) → (V). Preliminary work on paper chromatograms indicates a complex mixture, with broad bands occurring at all the expected positions. However, only hypoxanthine has been eluted and sufficiently purified for both spectrophotometric and chromatographic identification. Since the other expected intermediates are increasingly sensitive to ultraviolet radiation, their presence would be difficult to establish by ordinary chemical means. We are therefore irradiating C_{14}-labeled adenine and hypoxanthine for chromatographic examination.

One possible explanation of the inhibiting effect of oxygen on the ultraviolet decomposition of guanine and xanthine, is organic radical and peroxide formation, since oxygen is known to function frequently as an inhibitor in photochemical reactions involving radicals[12] by peroxide formation. Complex dependence on radiation intensity, such as the more reactive purines III, IV, V demonstrate, is also characteristic of photochemical decompositions involving radicals.

While chemical information is yet too fragmentary to provide a detailed mechanism of the ultraviolet decomposition of purines, any working hypothesis must be consistent with the following experimental observations. (1) The purines examined, except adenine under nitrogen, exhibit an initial induction period. (2) The second stage of breakdown is independent of purine concentration. (3) Hypoxanthine has been isolated from adenine irradiated both under oxygen and nitrogen.

Acknowledgment.—We are indebted to Prof. L. J. Heidt of the Massachusetts Institute of Technology for criticism of this manuscript and many helpful suggestions in connection with photochemical techniques, to Prof. E. Heinz of the Biochemistry Department, and to Dr. A. C. English of General Electric Company, for suggestions connected with the interpretation of kinetic data.

(11) The isolation of hypoxanthine from adenine irradiated under N_2 indicates that I → II may be a hydrolytic step, but the great difference in rates under O_2 and N_2 favors the oxidative sequence suggested above.

(12) E. J. Bowen, "The Chemical Aspects of Light," 2nd ed. Clarendon Press, Oxford, 1946, Chap. VI, pp. 205 ff. See also G. K. Rollefson and M. Burton, "Photochemistry and the Mechanism of Chemical Reactions," Prentice-Hall Inc., New York, N. Y., 1946, for scattered references to the role of oxygen in photochemical reactions—for example, pp. 165, 307–310, 381.

BOSTON, MASS.

[CONTRIBUTION FROM THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH

Studies on Diastereomeric α-Amino Acids and Corresponding α-Hydroxy Acids. IX. Configuration of the Isomeric γ-Hydroxyglutamic Acids

BY LEO BENOITON, MILTON WINITZ, SANFORD M. BIRNBAUM AND JESSE P. GREENSTEIN

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An epimeric mixture of the two racemic modifications of γ-hydroxyglutamic acid was prepared *via* the sodium ethoxide-catalyzed condensation of ethyl acetamidocyanoacetate with ethyl α-acetoxy-β-chloropropionate, followed by acid hydrolysis of the condensation product, so derived. Treatment of an aqueous solution of the epimeric mixture with hydrogen chloride gas to saturation led to the quantitative deposition of one racemate (A-form) as its lactone hydrochloride, whilst the other racemate (B-form) was isolated as the free amino acid upon adjustment of the filtrate to *p*H 3. That the A- and B-forms, so secured, were free from contamination of one by the other was evidenced by column chromatography on Dowex-1. After chloroacetylation of the A-form, as its lactone hydrochloride, and the B-form, as the free amino acid, the appropriate chloroacetyl-amino acid was subjected to asymmetric enzymic cleavage with cobalt-activated hog renal acylase I at *p*H 7.5. Such procedure permitted both the eventual isolation of each of the four optically pure stereomers and the establishment of the configuration of the α-asymmetric center of each, with $[M]^{26}D$ values in water as follows: L-A, −22.3°; D-A, +21.5°; L-B, +31.8°; D-B, −31.2°. Conversion of each of the optical isomers to its corresponding α,α′-dihydroxyglutaric acid was effected upon treatment with nitrous acid. The D- and L-enantiomorphs of A, so treated, and subsequently isolated as their respective barium salts, revealed $[M]^{26}D$ values of +31.4° and −30.9° (in water), respectively, whereas comparable treatment of the D- and L-forms of B led, in both instances, to the formation of *meso*-α,α′-dihydroxyglutaric acid which, by virtue of internal compensation, was devoid of optical activity. Such data established the stereochemical relationships between the α- and γ-asymmetric centers of each stereomer and permitted the assignment of a configurational designation to the γ-center of asymmetry of each.

γ-Hydroxyglutamic acid is a monoaminodicarboxylic acid which, by virtue of its two asymmetric centers, may exist as four optically active stereomers or two racemic modifications. Isolation of

this amino acid from plants was reported recently by Virtanen and Hietala,[1] but the paucity of material permitted determination of neither the optical rotation nor the stereochemical configuration of the natural product. Metabolism of the amino acid in the rat has been investigated[2] in the attempt to ascertain whether it may participate as a metabolic intermediate in the degradation of hydroxyproline.[3,4] Although the synthesis of an epimeric mixture of the four stereomers of γ-hydroxyglutamic acid has been achieved,[5] none of the optically active isomers has hitherto been characterized, nor has any description for the separation of the two diastereomeric racemates appeared. The present communication is primarily concerned with the separation of these diastereomeric racemates, the resolution of each racemic pair and the determination of the configuration of the α- and γ-asymmetric center of each of the optically active isomers.

Results and Discussion

Separation of the Racemic A- and B-Forms of γ-Hydroxyglutamic Acid.—An epimeric mixture comprising the two diastereoisomeric racemates of γ-hydroxyglutamic acid was prepared according to the directions of Benoiton and Bouthillier.[2-5] In essence, ethyl acetamidocyanacetate was condensed with ethyl α-acetoxy-β-chloropropionate, in the presence of sodium ethoxide, to yield the diethyl α-acetamido-α-cyano-γ-acetoxyglutarate. Acid hydrolysis of the latter compound yielded γ-hydroxyglutamic acid, which was precipitated upon the adjustment of the solution to *p*H 3 with lithium hydroxide, followed by the addition of ethanol thereto. The material was recrystallized only once from water–ethanol; additional recrystallization was avoided in order to obviate the danger of fractionation of diastereomers at this stage.

In a consideration of methods to be employed for the separation of the two racemic modifications constituting the above derived material, an attempt to exploit the possible differential solubilities of the hydrochloride salts of the two racemates, without prior derivatization, appeared particularly tempting. With this in mind, an aqueous solution of the epimeric mixture was saturated with hydrogen chloride gas in a manner reminiscent of that classically employed to precipitate glutamic acid hydrochloride from aqueous media. Upon chilling at −10° for 12–18 hr., some 45% of the starting material was deposited as a crystalline substance which analyzed for the lactone hydrochloride and to which the designation of A was assigned. That this material presumably represented a single, pure racemate was attested to by the fact that it precipitated, in nearly quantitative amount, upon treatment of its aqueous solution with hydrogen chloride, as above. The soluble racemate, which was designated as the B-form, could be isolated in good yield as the free amino acid upon evaporation of the original mother liquors to dryness, followed first by the adjustment of the residual material to *p*H 3 with lithium hydroxide and then by the addition of ethanol.[6] Repeated recrystallization of this material from either water–ethanol or water alone yielded beautiful rosettes which, however, invariably exhibited a positive flame test for lithium. An analytically pure product could nevertheless be secured readily by desalting with the mediation of a Dowex 1–acetate column, succeeded by elution of the amino acid with acetic acid.

The efficacy of the above procedure for the separation of the two racemic diastereomers could be readily assessed with the aid of column chromatography. Use was made of the fact that the original epimeric mixture undergoes complete resolution into two peaks[7] upon passage through the Dowex 1–acetate system of Hirs, Moore and Stein.[8] The effluent was collected in 10-ml. fractions and aliquots were analyzed for the presence of amino acid by the colorimetric ninhydrin method of Troll and Cannan.[9] A typical elution pattern is illustrated in Fig. 1. As chromatography of a 15-mg. sample of once-recrystallized lactone hydrochloride A, or once-recrystallized γ-hydroxyglutamic acid B, revealed but a single peak in each instance, the optical integrity of each racemate was demonstrated. In Fig. 1, which depicts the pattern

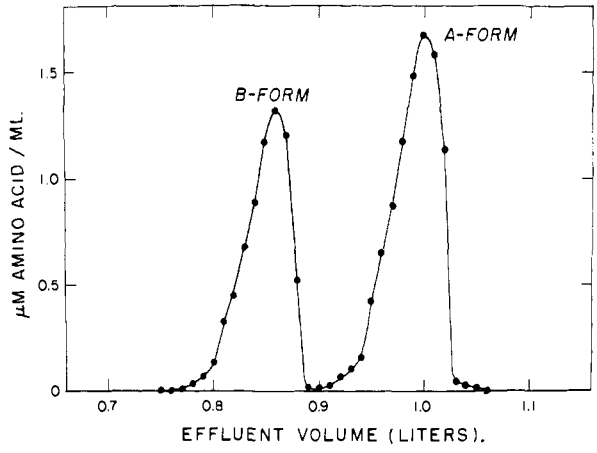

Fig. 1.—Separation of the diastereomers of γ-hydroxyglutamic acid (30 mg.) on a 2.5 × 35 cm. column of Dowex 1–acetate (200–400 mesh) eluted with 0.5 *N* acetic acid.

shown by an epimeric mixture of the A- and B-racemates, the identity of each peak as having arisen from either the former or the latter racemate was ascertained by re-chromatographing the combined eluates of the second peak, after the addition

(1) A. I. Virtanen and P. K. Hietala, *Acta Chem. Scand.*, **9**, 175 (1955).

(2) L. Benoiton and L. P. Bouthillier, *Can. J. Biochem. Physiol.*, **34**, 661 (1956).

(3) J. V. Taggart and R. B. Krakaur, *J. Biol. Chem.*, **177**, 641 (1949).

(4) R. Gianetto and L. P. Bouthillier, *Can. J. Biochem. Physiol.*, **32**, 154 (1954).

(5) L. Benoiton and L. P. Bouthillier, *Can. J. Chem.*, **33**, 1473 1955).

(6) An effort to crystallize the B-form as the lactone hydrochloride by precipitation with acetone from the original mother liquors failed to yield a solid material. In fact, titration of an aliquot of the solution with base afforded no indication that this form had even existed as the lactone.

(7) L. Benoiton, Ph.D. thesis, Department of Biochemistry, Univ. of Montreal, Montreal, Canada, 1956.

(8) C. H. Hirs, S. Moore and W. H. Stein, THIS JOURNAL, **76**, 6063 (1954).

(9) W. Troll and R. K. Cannan, *J. Biol. Chem.*, **200**, 803 (1953).

of 15 mg. of γ-hydroxyglutamic acid A thereto; both emerged as a single peak.

For the preparation of the derivatives of racemate A, described herein, it was found most convenient to employ the lactone hydrochloride without prior conversion to the free amino acid. Free γ-hydroxyglutamic acid A could be secured, however, upon treatment of an aqueous solution of the lactone hydrochloride with lithium hydroxide at room temperature, until the solution maintained a permanently alkaline *p*H. The crystalline amino acid deposited from solution upon adjustment of the *p*H to 3 with hydrochloric acid, followed by the addition of ethanol. In this case, recrystallization from water–ethanol sufficed to yield an analytically pure product. The racemates of both the A- and the B-forms of γ-hydroxyglutamic acid were isolated as their crystalline monohydrates.

Enzymic Resolution and Configuration of α-Asymmetric Centers.—During the past eight years, the general enzymic resolution procedures developed in this Laboratory[10,11] have been implicated in the procurement of the optically pure D- and L-isomers of some sixty different amino acids. As the procedures employed depended primarily upon the asymmetric cleavage of amino acid derivatives by purified hog kidney amidase[12] and acylase[13] fractions, the substrates invariably were confined to either the amide or N-acyl derivatives. In the present instance of the racemic A- and B-forms of γ-hydroxyglutamic acid, the choice of the action of the renal acylase I system upon the N-chloroacetyl derivatives of these racemates was governed, for the most part, by the prior knowledge that the closely related N-acylated glutamic acid[13,14] was highly susceptible to cleavage by this same enzyme system. However, initial determination of the rates of hydrolysis of the chloroacetyl derivatives of the A- and B-racemates, according to standard procedures,[13] revealed the disappointingly low values of 3 and 5 micromoles per hr. per mg. of protein nitrogen, respectively. In view of previous experience[15-17] that certain acylated amino acids exhibit a marked increase in their susceptibility to the hydrolytic action of acylase I in the presence of added cobalt ion, the applicability of such a phenomenon was here examined. A striking activation was indeed noted, with the hydrolytic rates being some ten times greater in the presence than in the absence of the metal ion. The degree of activation by cobalt ion, in this instance, represents the highest which has been observed for any substrate yet tested.[16] In any event, when the extremely low hydrolytic rates which were secured with the unactivated enzyme are borne in mind, the contribution rendered by cobalt ion in permitting a tenfold decrease in the amount of enzyme required, and thereby transforming an impracticable but feasible resolution procedure into a highly practicable one, may be fully appreciated.

The derivatives employed as substrates were prepared by the chloroacetylation of DL-A, as its lactone hydrochloride, and DL-B, as the free acid, under the usual Schotten–Baumann conditions. Since the chloroacetyl derivatives of both the A- and B-forms of γ-hydroxyglutamic acid exist as lactones, these required saponification with an excess of lithium hydroxide prior to digestion with the L-directed renal acylase I.[13] Enzymic digestion of a solution, which was 0.017 *M* with respect to the substrate and 10^{-3} *M* with respect to cobalt acetate,[16] was effected at *p*H 7.5 and 38°. The progress of the hydrolyses of the susceptible L-form was followed by the Van Slyke manometric ninhydrin–CO_2 procedure.[18] Upon completion of the reaction, the uncleaved chloroacetyl-D-amino acid was separated from the liberated L-amino acid *via* column chromatography on Dowex 50. Both the free D-amino acid, secured upon acid hydrolysis of the former compound, and the latter L-enantiomorph, which was generally contaminated with cobalt ion, were then desalted by absorption on a Dowex 1-acetate column, followed by elution with acetic acid. The pure D- and L-antipodes were secured upon evaporation of their respective eluates and subsequent crystallization of the residues from water–ethanol. Elemental analyses indicated that whilst the two antipodes of the B-form crystallized as the monohydrate, those of the A-form crystallized as the unsolvated amino acid. It might be reiterated that the racemates of both A- and B-forms are monohydrates, prior to resolution.

The molar rotation data for the four stereomers are presented in Table I. These values, determined in both water and 5 *N* hydrochloric acid, are equal and opposite for each enantiomorphic pair within the limits of experimental error.[19] In this connection, it is of interest to recall the observations of Clough[20] and of Lutz and Jirgensons,[21] that the exhibition of a more positive optical rotation value in acid than in water solutions appeared to be a general characteristic of certain L-amino acids. A previous paper of this series[22] has dem-

(10) J. P. Greenstein, *Advances in Protein Chem.*, **9**, 121 (1954).

(11) J. P. Greenstein, S. M. Birnbaum and M. C. Otey, *J. Biol. Chem.*, **204**, 307 (1953).

(12) D. Hamer and J. P. Greenstein, *ibid.*, **193**, 81 (1951); S. M. Birnbaum in "Methods in Enzymology," Vol. II, Academic Press, Inc., New York, N. Y., 1955, p. 397.

(13) S. M. Birnbaum, L. Levintow, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **194**, 455 (1952).

(14) L. Levintow, J. P. Greenstein and R. B. Kingsley, *Arch. Biochem. Biophys.*, **31**, 77 (1951).

(15) K. R. Rao, S. M. Birnbaum, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **198**, 507 (1952).

(16) R. Marshall, S. M. Birnbaum and J. P. Greenstein, THIS JOURNAL, **78**, 4636 (1956).

(17) R. Marshall, S. M. Birnbaum, M. Winitz and J. P. Greenstein, *ibid.*, **79**, 4538 (1957).

(18) One molecular equivalent of γ-hydroxyglutamic acid liberates about 14% more than the theoretical amount of carbon dioxide during the manometric ninhydrin–CO_2 procedure, presumably due to some decomposition of the γ-carboxyl group.

(19) Unfortunately, since neither the L- nor the D-isomers of the A- and B-forms were susceptible to the action of either L-amino acid oxidase (*Crotalus adamanteus*) or hog renal D-amino acid oxidase, their optical purity could not be tested (*cf.* A. Meister, L. Levintow, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **192**, 535 (1951)). However, in view of previous experience with a number of other amino acids which have been resolved through the mediation of acylase I, the optical purity of the stereomers so secured is, in all likelihood, 99.9% or greater.[10]

(20) G. W. Clough, *J. Chem. Soc.*, **107**, 1509 (1915).

(21) O. Lutz and B. Jirgensons, *Ber.*, **63**, 448 (1930); **64**, 1221 (1931); **65**, 784 (1932).

(22) M. Winitz, S. M. Birnbaum and J. P. Greenstein, THIS JOURNAL, **77**, 716 (1955).

TABLE I

CONTRIBUTION OF THE ASYMMETRIC α- AND γ-CARBON ATOMS TO THE MOLAR ROTATION OF THE DIASTEREOMERIC γ-HYDROXYGLUTAMIC ACIDS[a]

Amino acid	$[M]^{26}D$ In H_2O	$[M]^{26}D$ In 5 *N* HCl	Molar rotation of α in H_2O	α in 5 *N* HCl	$\alpha_{HCl} - \alpha_{H_2O}$	γ in H_2O	γ in 5 *N* HCl	$\gamma_{HCl} - \gamma_{H_2O}$
L-γ-Hydroxyglutamic acid (A)	−22.3°	+ 5.0°	+4.8	+33.3	+28.5	−26.8	−28.4	−1.6
D-γ-Hydroxyglutamic acid (A)	+21.5	− 5.0	−4.9	−33.4	−28.5	+26.7	+28.3	+1.6
L-γ-Hydroxyglutamic acid (B)	+31.8	+61.6	+4.8	+33.3	+28.5	+26.7	+28.3	+1.6
D-γ-Hydroxyglutamic acid (B)	−31.2	−61.8	−4.9	−33.4	−28.5	−26.8	−28.4	−1.6

[a] Rotations performed in a 2-dcm. tube at a concentration of 1%.

onstrated, in addition, that the Clough–Lutz–Jirgensons rule was generally valid with amino acids which incorporated more than a single center of asymmetry only if the H_2O-to-HCl shift was based upon the calculated contribution (partial rotation) of the α-asymmetric center, rather than upon the observed rotation. Extension of such calculations[22] to the stereomers of γ-hydroxyglutamic acid, as given in Table I, reveal a positive H_2O-to-HCl shift in the partial rotations of the α-asymmetric centers of both L-forms, in concordance with this concept.

In the search for alternative resolution procedures, investigation of the susceptibility of the diastereomeric γ-hydroxyglutamic acids, and several derivatives thereof, to other enzymic systems was also effected. Utilization of the method of asymmetric oxidative destruction of a single antipode proved unsuccessful by virtue of the lack of susceptibility of both the A- and B-forms of the amino acid to the oxidative action of L-amino acid oxidase (*Crotalus adamanteus*) and hog renal D-amino acid oxidase. Acylase II, for which the only known substrates to date are various acylated L-aspartic acids,[13] exhibited no tendency to hydrolyze the chloroacetyl-γ-hydroxyglutamic acids. Trifluoroacetyl-γ-hydroxyglutamic acid B, on the other hand, although cleaved by acylase I at approximately five times the rate of the corresponding chloroacetyl derivative, was not suitable as a substrate in resolutions by virtue of the lability of the trifluoroacetyl group during prolonged periods of incubation at *p*H 7.5. An unanticipated phenomenon was encountered when the α-amide of γ-hydroxyglutamic acid[23] (γ-hydroxyisoglutamine) was subjected to the hydrolytic action of hog kidney amidase.[12] It was noted that a solution of the amide, containing added magnesium ion, was rapidly hydrolyzed to the free amino acid upon incubation at *p*H 8, either in the presence or absence of enzyme; spontaneous decomposition of γ-hydroxyisoglutamine also occurred in the absence of added magnesium ion but at a markedly lesser rate.

(23) The γ-hydroxyisoglutamines employed in the present study were prepared by ammonolysis of the corresponding carbobenzoxylactone, followed by hydrogenolysis of the carbobenzoxylated α-amide, so derived. In the case of the B-form, where carbobenzoxylation of the free amino acid under Schotten–Baumann conditions led to the ultimate isolation of the carbobenzoxyamino acid rather than the lactone, the compound could be lactonized upon refluxing in toluene, with the liberated water being removed azeotropically. It is of interest to note, in this connection, that whilst the chloroacetyl, trifluoroacetyl and carbobenzoxy derivatives of the A-form and the chloroacetyl derivative of the B-form of γ-hydroxyglutamic acid are all isolated as their lactone derivatives subsequent to acylation, the trifluoroacetyl and carbobenzoxy derivatives of the B-form are secured as the free amino acids under the same conditions.

Configuration of the γ-Asymmetric Centers.—As was demonstrated above, the use of the L-directed acylase I permitted the assignment of an L- or D-configuration to the α-carbon atom of each of the four stereomers of γ-hydroxyglutamic acid. In order to establish the configuration of the γ-asymmetric centers, a sample of each optically active stereomer was converted to the corresponding α,α′-dihydroxyglutaric acid isomer upon deamination with nitrous acid. That such conversion of an α-amino acid to the corresponding α-hydroxy acid proceeds with retention of configuration and without appreciable racemization has been demonstrated by kinetic studies[24,25] and, more recently, through the application of stereospecific α-hydroxy acid oxidases.[26,27] The α-configuration of each of the isomers of α,α′-dihydroxyglutaric acid can thereupon be considered as identical to that of the α-amino acid from which it was derived. The $[M]^{26}D$ values for the two α,α′-dihydroxyglutaric isomers corresponding to the D- and L-antipodes of γ-hydroxyglutamic acid A, measured as 1% solutions of the barium salts in water, were +31.4° and −30.9°, respectively. On the other hand, the two isomers of α,α′-dihydroxyglutaric acid, whose parent amino acids represented the antipodes of γ-hydroxyglutamic acid B, were completely devoid of optical activity.

An examination of the Fischer projections of the four stereomers of γ-hydroxyglutamic acid and the α,α′-dihydroxyglutaric acid isomers derived therefrom, as revealed in Fig. 2, will readily explain the above results. It can readily be seen that although the dihydroxy acid molecule embodies two asymmetric centers, it also possesses a symmetry such that of the four theoretically possible isomers, only two constitute an externally compensated optically active pair, whilst the other two, by virtue of internal compensation, constitute the identical optically inactive *meso*-form. Since the two antipodes of the B-form of γ-hydroxyglutamic acid gave rise to the *meso*-form of α,α′-dihydroxyglutaric acid, then their α-amino and γ-hydroxyl groups must assume a *cis* representation in the Fischer formulation. The corresponding functional groups of the

(24) Kinetic studies (P. Brewster, F. Hiron, E. D. Hughes, C. K. Ingold and P. A. D. S. Rao, *Nature*, **166**, 178 (1950)) have demonstrated that, except in the presence of an α-phenyl substituent, the nitrous acid mediated deamination of an α-amino acid proceeds *via* an Sn1 mechanism, wherein no inversion of configuration occurs by virtue of the presence of the configuration-holding α-carboxylate ion.

(25) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1953.

(26) C. G. Baker, *Arch. Biochem. Biophys.*, **41**, 325 (1952).

(27) M. Winitz, L. Bloch-Frankenthal, N. Izumiya, S. M. Birnbaum, C. G. Baker and J. P. Greenstein, THIS JOURNAL, **78**, 2423 (1956).

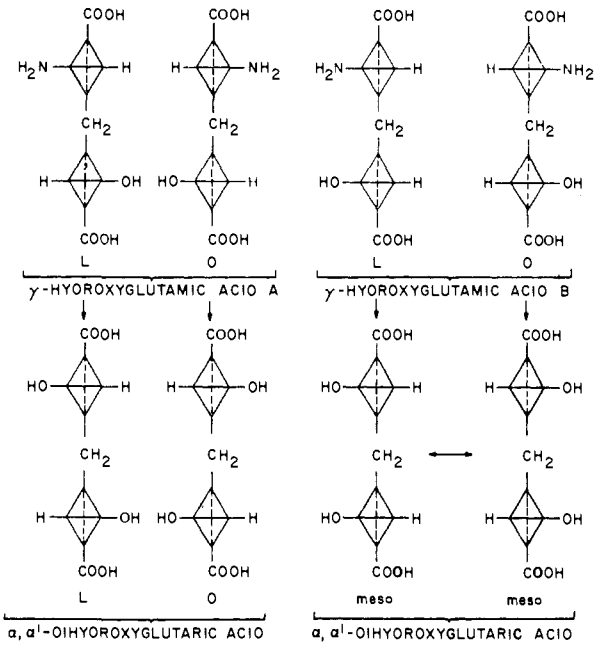

Fig. 2.—Fischer projections of the four stereomers of γ-hydroxyglutamic acid and the corresponding α,α'-dihydroxyglutaric acids derived therefrom.

enantiomorphic A-forms, on the other hand, are represented in the *trans* position. Since the configuration of the α-asymmetric carbon atoms of each of the γ-hydroxyglutamic acids previously was obtained from enzymic data, then the configuration of the γ-asymmetric center of each stereomer relative to L-serine or D-glyceraldehyde, as the standards of reference of the amino acid and carbohydrate series, respectively, then becomes known.[28] On this basis, an L_s- or D_g-configuration may be assigned to the γ-asymmetric center of L-γ-hydroxyglutamic acid A and D-γ-hydroxyglutamic acid B, whereas the corresponding asymmetric center of their respective enantiomorphs assumes a D_s- or L_g-configuration.[28]

Sufficient information is provided by the above discussion to permit the stereochemical correlation of each of the four stereomers of γ-hydroxyglutamic acid with those of the corresponding γ-hydroxyprolines. The correct Fischer representations for each of the hydroxyproline isomers became known in consequence of the investigations of Neuberger,[29] who established the configuration of the γ-asymmetric center of L-hydroxyproline, and of Kaneko[30] and Karrer and Portmann,[31] who related this latter amino acid to L-proline and L-glutamic acid, respectively.[32] As is revealed in Fig. 3, L-hydroxyproline (I) thereupon may be configurationally correlated with L-γ-hydroxyglutamic acid B (II), whilst L-allohydroxyproline (III) becomes the

(28) H. B. Vickery, *J. Biol. Chem.*, **169**, 237 (1947). A noteworthy discussion of the confusion which may arise with regard to configurational designations in molecules wherein two or more asymmetric centers may serve as the point of reference has been presented by H. B. Vickery (*Science*, **113**, 314 (1951)).

(29) A. Neuberger, *J. Chem. Soc.*, 429 (1945).

(30) Y. Kaneko, *J. Chem. Soc. Japan*, **61**, 207 (1940).

(31) P. Karrer and P. Portmann, *Helv. Chim. Acta*, **31**, 2088 (1948).

(32) *Cf.* C. S. Hudson and A. Neuberger, *J. Org. Chem.*, **15**, 24 (1950).

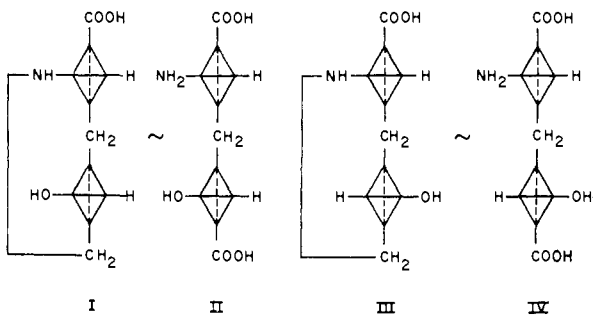

Fig. 3.—Stereochemical equivalence of the L- and L-alloforms of γ-hydroxyglutamic acid with the corresponding forms of γ-hydroxyproline.

stereochemical equivalent of L-γ-hydroxyglutamic acid A (IV). With this in mind, it now becomes possible to eliminate the A- and B-designations for the diastereomeric γ-hydroxyglutamic acids and adopt the prefix allo- in their stead[28] (Table II).

TABLE II

CONFIGURATIONAL DESIGNATION AND NOMENCLATURE OF THE ISOMERIC γ-HYDROXYGLUTAMIC ACIDS

Isomer	Configuration of γ-asymmetric center	Recommended designation
L-γ-Hydroxyglutamic acid A	L_s or D_g	L-Allo-γ-hydroxyglutamic acid
D-γ-Hydroxyglutamic acid A	D_s or L_g	D-Allo-γ-hydroxyglutamic acid
L-γ-Hydroxyglutamic acid B	D_s or L_g	L-γ-Hydroxyglutamic acid
D-γ-Hydroxyglutamic acid B	L_s or D_g	D-γ-Hydroxyglutamic acid

The direct chemical transformation of L-glutamic acid to L-proline, by Pravda and Rudinger,[33] suggests the feasibility of analogous conversion of each of the stereomers of γ-hydroxyglutamic acid into the corresponding hydroxyproline isomers and thus makes reasonable the adoption of a designation which is consistent for both.

Experimental

γ-Hydroxyglutamic Acid (Epimeric Mixture).—The sodium condensation of ethyl α-acetoxy-β-chloropropionate[34,35] with ethyl acetamidocyanoacetate, as described by Benoiton and Bouthillier,[2,5] was employed. After hydrolysis of the condensation product with 20% hydrochloric acid, the amino acid was isolated from the hydrolyzate upon adjustment to *p*H 3 with lithium hydroxide, followed by the addition of alcohol. The yield of once-recrystallized material, from water–alcohol, was 50–60%.

Separation of the Racemic A- and B-Forms of γ-Hydroxyglutamic Acid.—Eighty grams of the epimeric mixture was dissolved in 500 ml. of dilute hydrochloric acid and the cooled solution saturated with hydrogen chloride. The solution was kept at −10° overnight and the precipitate filtered and washed first with cold concentrated hydrochloric acid, then with acetone. Two additional crops were obtained by working up the combined filtrate and washings in comparable manner. The combined products were recrystallized from 200 ml. of hydrogen chloride-saturated water. Analysis of the substance, dried over sodium hydroxide pellets in a vacuum desiccator, was consistent with that of the lactone hydrochloride. The yield of DL-γ-hydroxyglutamic acid A lactone hydrochloride was 45% of theory (based on weight of starting material). All derivatives of the A-

(33) Z. Pravda and J. Rudinger, *Collection Czechoslov. Chem. Communs.*, **20**, 1 (1955).

(34) C. F. Koelsch, THIS JOURNAL, **52**, 1105 (1930).

(35) W. O. Kenyon, C. C. Unruh and T. T. M. Laakso, U. S. Patent 2,499,393 (1950).

form of hydroxyglutamic acid, subsequently described, were prepared from this material.

Anal. Calcd. for $C_5H_8O_4NCl$: C, 33.1; H, 4.4; N, 7.7; Cl, 19.5. Found: C, 33.0; H, 4.4; N, 7.6; Cl, 19.8.

The filtrate remaining after the precipitation of the A-form as the lactone hydrochloride was concentrated to an oil and the concentration repeated several times after the addition of water in order to remove the excess hydrochloric acid. The residue was dissolved in 300 ml. of water and the *p*H first raised to 8 with lithium hydroxide, then adjusted to 3 with hydrochloric acid. Addition of ethanol, followed by cooling, led to the deposition of free DL-γ-hydroxyglutamic acid B. The product, recrystallized from water–alcohol (yield 40%) contained traces of lithium which could not be removed by further recrystallization. This material was, however, satisfactory for the preparation of the derivatives of γ-hydroxyglutamic acid B. Analytically pure material was readily obtained by placing an aqueous solution of 3 g. thereof on a 2.5 × 35 cm. column of Dowex 1–acetate (50–100 mesh), previously washed with water. After the eluate volume of water had reached 200 ml., the amino acid was eluted with 0.5 *N* acetic acid. Evaporation of the eluate, followed by crystallization of the residue from water–ethanol, yielded DL-γ-hydroxyglutamic acid B monohydrate as fine needles.

Anal. Calcd. for $C_5H_9O_5N \cdot H_2O$: C, 33.2; H, 6.1; N, 7.7. Found: C, 33.2; H, 6.2; N, 7.7.

Treatment of a sample of the lactone hydrochloride of the A form with lithium hydroxide to a basic *p*H, followed by readjustment to *p*H 3 with lithium hydroxide and the subsequent addition of ethanol, yielded analytically pure DL-γ-hydroxyglutamic acid A monohydrate. Column purification was not necessary in this case.

Anal. Calcd. for $C_5H_9O_5N \cdot H_2O$: C, 33.2; H, 6.1; N, 7.7. Found: C, 33.2; H, 6.3; N, 7.7.

Chloroacetyl-DL-γ-hydroxyglutamic Acid A Lactone.—Thirty-six grams of DL-γ-hydroxyglutamic acid A lactone was dissolved in 150 ml. of 4 *N* NaOH and the solution cooled to 5° in an ice-bath. To the stirred solution was added, in alternate portions, 16 ml. of chloroacetyl chloride and 75 ml. of 4 *N* NaOH. Stirring was continued for several hours, after which time the solution was adjusted to *p*H 1.7 with 4 *N* HCl. The solution was extracted twice with four volumes of ethyl acetate, concentrated *in vacuo* until sodium chloride separated out and again extracted with ethyl acetate. The combined extracts were dried over sodium sulfate, concentrated *in vacuo* to an oil, the latter taken up in a small volume of acetone and the resulting solution treated with ether to incipient turbidity. Scratching of the sides of the vessel, followed by cooling, resulted in crystallization. The product was filtered, washed with ether and then recrystallized from acetone–ether; yield 9 g. (20%), m.p. 183–185°.

Anal. Calcd. for $C_7H_8O_5NCl$: C, 37.9; H, 3.6; N, 6.3; Cl, 16.0. Found: C, 38.1; H, 3.7; N, 6.3; Cl, 15.8.

Chloroacetyl-DL-γ-hydroxyglutamic acid B lactone was prepared in an identical manner from 27 g. of DL-γ-hydroxyglutamic acid B dissolved in 75 ml. of 4 *N* NaOH; yield 11 g. (33%), m.p. 172–173°.

Anal. Calcd. for $C_7H_8O_5NCl$: C, 37.9; H, 3.6; N, 6.3; Cl, 16.0. Found: C, 38.1; H, 3.9; N, 6.4; Cl, 16.1.

Carbobenzoxy-DL-γ-hydroxyglutamic Acid A Lactone.—Nine grams of DL-γ-hydroxyglutamic Acid A lactone and 21 g. of sodium bicarbonate were dissolved in 125 ml. of water. To the cooled solution was added, in small portions, 9 ml. of carbobenzoxy chloride, with intermittent vigorous shaking and cooling. The reaction mixture was then placed on a mechanical shaker for 2 hr., after which time a fluffy white precipitate was present. The mixture was extracted once with petroleum ether and the aqueous layer, upon which the precipitate floated, acidified with 4 *N* HCl. The fine white powder which deposited was filtered over suction and washed with cold water. The material (11 g.) was suspended in 100 ml. of water, brought into solution by the addition of sodium carbonate and precipitated by the dropwise addition of 4 *N* HCl. The product was collected and washed with water until the washings were chloride free; yield 10 g. (70%), m.p. 165–167°. A sample, recrystallized from ethyl acetate, melted at 168–169°.

Anal. Calcd. for $C_{13}H_{13}O_6N$: C, 55.9; H, 4.7; N, 5.0. Found: C, 55.9; H, 4.8; N, 5.0.

Carbobenzoxy-DL-γ-hydroxyglutamic Acid B.—This compound was prepared, in a manner similar to the corresponding A-form, from 18 g. of DL-γ-hydroxyglutamic acid B, 33.6 g. of sodium bicarbonate and 18 ml. of carbobenzoxy chloride. As in the previous case, a copious white precipitate was present after the reaction. However, the material dissolved upon acidification of the reaction mixture and no other precipitate or oil separated out. The solution was thereupon exhaustively extracted with ethyl acetate and the extract dried over sodium sulfate and evaporated to an oil. The residue, which solidified upon cooling, was dissolved in hot ethyl acetate (250 ml.), after which time the solution was concentrated in a jet of dry air. The product was filtered and washed with cold ethyl acetate; yield 12 g. (40%), m.p. 132–133°.

Anal. Calcd. for $C_{13}H_{15}O_7N$: C, 52.5; H, 5.1; N, 4.7. Found: C, 52.8; H, 5.2; N, 4.8.

Carbobenzoxy-DL-γ-hydroxyglutamic Acid B Lactone.—A suspension of 9 g. of carbobenzoxy-DL-γ-hydroxyglutamic acid B in 500 ml. of toluene was refluxed in a flask provided with a Dean–Stark tube and condenser. After 3 hr., the theoretical amount of water had been distilled over. The refluxing was continued overnight, after which time the solution was evaporated to dryness. A sample, recrystallized from ethyl acetate, melted at 152–153°.

Anal. Calcd. for $C_{13}H_{13}O_6N$: C, 55.9; H, 4.7; N, 5.0. Found: C, 55.5; H, 4.7; N, 5.0.

Carbobenzoxy-DL-γ-hydroxyisoglutamine A (Ammonium Salt).—Eight grams of carbobenzoxy-DL-γ-hydroxyglutamic acid A lactone was added to 100 ml. of methanol which had been saturated previously with dry ammonia at 0°. The container, a 500-ml. reagent bottle, was sealed with a rubber stopper held firmly in place by wire and left at room temperature for 48 hr. After removing the stopper, the solution was left under the hood for several hours, after which time the whole solidified into a fluffy white cake. The product was filtered with the aid of ether, sucked dry and recrystallized from hot methanol–ether; yield 7 g. (80%), m.p. 160–161°.

Anal. Calcd. for $C_{13}H_{19}O_6N_3$: C, 49.8; H, 6.1; N, 13.4. Found: C, 49.8; H, 6.1; N, 13.4.

Carbobenzoxy-DL-γ-hydroxyisoglutamine B (ammonium salt) was obtained in the same manner as the A-form from its corresponding lactone, m.p. 149–150°.

Anal. Calcd. for $C_{13}H_{19}O_6N_3$: C, 49.8; H, 6.1; N, 13.4. Found: C, 49.6; H, 6.2; N, 13.4.

DL-γ-Hydroxyisoglutamine A and B.—The ammonium salt of the appropriate carbobenzoxy-γ-hydroxyisoglutamine (A- or B-form) was hydrogenated in an aqueous solution, previously made acid with acetic acid, for 18 hr. in the presence of palladium black catalyst. After removal of the catalyst, the solution was evaporated to dryness at a temperature not exceeding 40° and the residual material dissolved in warm water (75 ml./g.). An equal volume of alcohol was added and the solution cooled overnight. The product which deposited was filtered over suction and recrystallized from water–alcohol (fine, flocculent needles). Yields were practically quantitative.

Anal. Calcd. for $C_5H_{10}O_4N_2$: C, 37.0; H, 6.2; N, 17.3. Found (Form A): C, 36.7; H, 6.2; N, 17.1. Found (Form B): C, 36.8; H, 6.0; N, 17.1.

Trifluoroacetyl-DL-γ-hydroxyglutamic acid A lactone was prepared according to the procedure of Schallenberg and Calvin.[36] To a solution of 5.4 g. of DL-γ-hydroxyglutamic acid A lactone hydrochloride and 7.6 g. of sodium bicarbonate in 30 ml. of water was added 5.2 ml. of ethyl thiolfluoroacetate[37] and the reaction mixture mechanically shaken for 24 hr. The solution was cooled in an ice-bath, acidified with concentrated hydrochloric acid and then extracted with ethyl acetate. The sodium sulfate-dried extract was evaporated to an oil which soon solidified. Recrystallization was effected from ethyl acetate–benzene; yield 2 g., m.p. 195–196°.

Anal. Calcd. for $C_7H_8O_5NF_3$: C, 34.9; H, 2.5; N, 5.8. Found: C, 34.7; H, 2.7; N, 5.8.

(36) E. F. Schallenberg and M. Calvin, THIS JOURNAL, **77**, 2779 (1955).

(37) M. Hauptschein, C. S. Stokes and E. A. Nodiff, *ibid.*, **74**, 4005 (1952).

Trifluoroacetyl-DL-γ-hydroxyglutamic acid B was prepared in the same manner as the corresponding A-form from 5.4 g. of amino acid and 5 g. of sodium bicarbonate; yield 3 g., m.p. 190° (softens at 138°).

Anal. Calcd. for $C_7H_8O_6NF_3$: C, 32.4; H, 3.1; N, 5.4. Found: C, 32.7; H, 3.1; N, 5.5.

Resolution of Chloroacetyl-DL-γ-hydroxyglutamic Acid A and B.—Five and one-half grams of chloroacetyl-DL-γ-hydroxyglutamic acid lactone (A- or B-form) was suspended in 200 ml. of water and brought into solution by the addition of lithium hydroxide until the *p*H remained constant at 8.5–9. Addition of 375 mg. of cobalt acetate and 500 mg. of purified acylase I powder[13] (300 mg. in the case of diastereomer B) lowered the *p*H to about 7.5. The reaction mixture was diluted to 1500 ml. (0.017 *M* substrate, 10^{-3} *M* cobalt) and incubated at 38°, with the *p*H of the medium being readjusted up to 7.5 whenever required during the first few hours of digestion. After 24 hr., analysis on an aliquot by the usual manometric ninhydrin-CO_2 procedure revealed that the hydrolysis had proceeded to 50%.[15] An additional 100 mg. of enzyme was added and the incubation allowed to proceed another 12 hr. The solution was then deproteinized by adjusting the *p*H to 5 and filtering with the aid of charcoal. The filtrate was concentrated to about 100 ml. and placed on a 2.5 × 35 cm. column of Dowex 50 (100–200 mesh) which was in the acid form and previously had been washed with water; 400 ml. of water was allowed to flow through the column, followed by *N* HCl. The emergence of the chloroacetyl-D-amino acid in the first 200 ml. of aqueous eluate was indicated by the low *p*H of the solution. The appearance of the L-amino acid in the hydrochloric acid eluate was revealed by spot test employing a basic ninhydrin solution. The fractions containing the chloroacetyl-D-component were combined, concentrated hydrochloric acid added to a final concentration of 2.5 *N* and the solution refluxed for 3 hr. to liberate the D-amino acid.

The two solutions containing the free enantiomorphs were evaporated to dryness in order to remove excess hydrochloric acid, the residues were dissolved in water and the solutions made alkaline with sodium hydroxide and subsequently desalted by passage through a 2.5 × 35 cm. column of Dowex 1 (50–100 mesh) in the acetate form (previously washed with water). The cations, including some cobalt in the case of L-enantiomorph, were washed through with water and the amino acid then eluted with 0.5 *N* acetic acid. Complete removal of the acetic acid from the appropriate eluate by repeated evaporation yielded crystalline material in the case of both A-antipodes and an oil in the case of the B-antipodes. These residues were dissolved in hot water, the solutions decolorized with charcoal and ethanol added. After cooling, the products were recovered by filtration and recrystallized from hot water by the addition of a small volume of ethanol. The final products were washed first with ethanol, then acetone and finally dried over phosphorus pentoxide *in vacuo*.

The optically active enantiomorphs of the A-form crystallized as large transparent prisms, whilst those of the B-form were secured as small, irregular, crystalline needles. Two resolutions were effected with each racemate. The yields varied from 40 to 60%; optical rotation data appear in Table I.

Anal. Calcd. for $C_5H_9O_5N$: C, 36.8; H, 5.6; N, 8.6. Found (L-A): C, 36.7; H, 5.7; N, 8.6. Found (D-A): C, 36.8; H, 5.8; N, 8.5. Calcd. for $C_5H_9O_5N \cdot H_2O$: C, 33.2; H, 6.1; N, 7.7. Found (L-B): C, 33.4; H, 6.1; N, 7.8. Found (D-B): C, 33.5; H, 6.2; N, 7.8.

Nitrous Acid Deamination of γ-Hydroxyglutamic Acid to α,α'-Dihydroxyglutaric Acid.—Two hundred and fifty mg. of each of the four optically active isomers of γ-hydroxyglutamic acid was dissolved in a mixture of 1.5 ml. of *N* HCl, 3 ml. of acetic acid and 6 ml. of water. To the cooled solution was added, in small portions with shaking, a solution of 1.2 g. of sodium nitrite in 2 ml. of water.[27] The mixture was stored at 5° for 1 hr. and then at room temperature overnight. Three ml. of concentrated hydrochloric acid was added, the solution evaporated to dryness and the evaporation repeated several times after the addition of water. The residue was extracted three times with boiling acetone, the sodium sulfate-dried extract concentrated to an oil and the latter taken up in water and shaken in the presence of a few grams of Dowex 50 (20-50 mesh) to remove any residual amino acid or sodium ions. The aqueous solution was treated with barium hydroxide solution to a *p*H of 9 (to hydrolyze any possible lactone) and the *p*H subsequently readjusted to 5 by the addition of a few beads of Dowex 50. To the final solution (75 ml.) was added three volumes of acetone with vigorous shaking. The barium salt was filtered, triturated with acetone, collected by centrifugation and dried at 137° *in vacuo* over phosphorus pentoxide to remove final traces of water; yield 250 mg.; $[M]^{26}D$ values, as 1% solutions in water, were: L-A, −30.9°; D-A, +31.4°; L-B, 0°; D-B, 0°. *Anal.* Calcd. for $C_5H_8O_5Ba$: Ba, 45.8. Found: L-A, Ba, 45.6; D-A, 45.5; L-B, Ba, 45.6; D-B, 45.7.

BETHESDA 14, MARYLAND

[CONTRIBUTION FROM THE ANNEX C LABORATORY, DEPARTMENT OF CHEMISTRY, EMORY UNIVERSITY]

The Reaction of N-Benzylpyrrole with Acetylenedicarboxylic Acid. A Diels–Alder Addition to a Pyrrole[1]

BY LEON MANDELL AND WESLEY A. BLANCHARD[2]

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The reaction of N-benzylpyrrole and acetylenedicarboxylic acid has been shown to lead to the formation of three products, I, II and III. Compound I represents the first case of a pyrrole partaking in a normal Diels–Alder addition. The proof of structure of these products is discussed.

Although a great number of different types of dienes have been shown to be capable of participating in the Diels–Alder reaction,[3] including the diene system of heterocycles such as furans[4] and thiophenes,[5,6] the dienic moiety of pyrroles has in general resisted normal Diels–Alder addition[7] and undergoes instead substitutive processes leading to α-substituted pyrroles[8] and dihydroindoles.[9] As part of a synthetic scheme designed to utilize this known mode of reaction of pyrroles with dienophiles, the reaction of N-benzylpyrrole and acetylenedicarboxylic acid was investigated.

Reaction between N-benzylpyrrole and acetylenedicarboxylic acid was effected readily by allowing an ether solution of the two to reflux for 24

(1) For a preliminary account of this work, see L. Mandell and W. A. Blanchard, THIS JOURNAL, **79**, 2343 (1957).

(2) Taken in part from a dissertation submitted by W. A. Blanchard in partial fulfillment of the requirements for the Ph.D. degree at Emory University.

(3) For a review of the Diels–Alder reaction see M. C. Kloetzel and H. L. Holmes, in R. Adams, Ed., "Organic Reactions," Vol. IV, John Wiley and Sons, Inc., New York, N. Y., 1948.

(4) O. Diels and K. Alder, *Ber.*, **62**, 554 (1929).

(5) D. B. Clapp, THIS JOURNAL, **61**, 2733 (1939).

(6) C. F. H. Allen and J. W. Gates, *ibid.*, **65**, 1283 (1943).

(7) An interesting exception to this is the recent report of G. Wittig, *Angew. Chem.*, **69**, 245 (1957).

(8) O. Diels and K. Alder, *Ann.*, **498**, 1 (1932).

(9) O. Diels and K. Alder, *ibid.*, **490**, 267 (1931).