β-Hydroxynorleucine: Separation of Its Isomers and Biological Studies

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Abstract □ Separation of the four isomers of β-hydroxynorleucine was accomplished by partition column chromatography and asymmetric enzymatic hydrolysis of the *N*-chloroacetyl derivatives. From these, the corresponding *N*-chloroacetyl derivatives were made. The purity and configuration of each isomer of the free acid and *N*-chloroacetylated derivative were ascertained by: (*a*) paper chromatography in five solvent systems, (*b*) elemental analysis, (*c*) Van Slyke nitrous acid determination of primary amino nitrogen, (*d*) Van Slyke ninhydrin determination of α-carbonyl carbon, and (*e*) optical rotation. Comparison of the rate of enzymatic hydrolysis by hog renal acylase I of the *N*-chloroacetyl derivative of the L-isomers of each diastereomer showed that the acyl B isomer is a better substrate than the acyl A isomer, where A denotes the faster moving diastereomer and B denotes the slower moving diastereomer in a defined chromatographic solvent system. Microbiological assay using *Lactobacillus casei* in a system selected for screening for possible antitumor activity indicated that while none of the isomers as free amino acids had any growth inhibitory action, the *N*-acylated isomers showed modest but significant activity. The *N*-chloroacetyl derivative of the D-enantiomorph of diastereomer B exhibited the greatest growth inhibitory activity, showing about twice the activity of the other three isomers.

Keyphrases □ β-Hydroxynorleucine—isomers separated, *N*-chloroacetyl derivatives prepared, enzymatic hydrolysis and microbiological growth inhibitory activity of isomers compared □ Enantiomers—β-hydroxynorleucine, separation, comparison of biological activity □ Hydrolysis, enzymatic—isomers of β-hydroxynorleucine compared □ Microbiological assay—growth inhibitory activity of isomers of β-hydroxynorleucine compared

By using a general method for the chemical synthesis of β-hydroxy-α-amino acids, as described previously, 10 β-hydroxy-α-amino acids have been prepared thus far (1). When these amino acids were screened for probable antitumor activity by a microbiological method (2), it was noted that the *N*-chloroacetyl derivatives of β-hydroxyleucine and of β-hydroxynorleucine exhibited modest but significant degrees of inhibitory action (3).

The compounds as chemically prepared are racemic and probably diastereomeric mixtures; therefore, any biological activity exhibited by them would be the net effect of the action of each of the four isomers present, be it growth inhibitory, growth stimulatory, or inactive. The purposes of this article are to: (*a*) describe the isolation of the four isomers of each of these amino acids and the preparation of the corresponding *N*-acyl derivatives, and (*b*) report the findings of the study of the biological properties of these isomers using microbial systems.

EXPERIMENTAL

β-Hydroxynorleucine[1] was prepared as described previously (1).

The separation of the diastereomers was accomplished by means of partition column chromatography, with cellulose powder as the immobile phase. The solvent system consisted of methyl ethyl ketone–1-butanol–concentrated ammonia–water (3:5:1:1) (6) as described for the separation of the diastereomers of β-hydroxyhomomethionine and of β-hydroxymethoxinine (7). The resolution of the optical isomers was accomplished by use of hog renal acylase, as described by Greenstein (8).

RESULTS

Separation of Diastereomers of β-Hydroxynorleucine—A typical procedure for the separation of the diastereomers of β-hydroxynorleucine, similar to that described for the separation of the diastereomers of β-hydroxyhomomethionine and of β-hydroxymethoxinine (see Ref. 7 for details) is outlined here.

DL-β-Hydroxynorleucine AB[2] (250 mg, 1.69 mmoles) was dissolved in 4 ml of the methyl ethyl ketone solvent system, and the resulting solution was applied to a 25 × 150-cm column containing cellulose powder[3], which had been poured into the column as a slurry of the solvent system[4]. The column was eluted with the methyl ethyl ketone system at a flow rate of 0.2 ml/min at room temperature (22–25°)[5].

Three-milliliter fractions were collected. The fractions appearing between 309 and 343, 344 and 384, and 385 and 472 ml, containing isomer A, a mixture of isomers A and B, and isomer B, respectively, were collected and isolated from the solutions as described (7). The yields were 98.2 mg of isomer A (39.5%) and 94.0 mg of isomer B (37.5%), based on the initial weight of the mixture of the isomers. The unseparated portion, containing a mixture of isomers A and B, was similarly isolated by alcohol precipitation of the concentrated aqueous solution. The product was passed through a second column for further separation of the diastereomers.

The respective isomers yielded single spots when chromatographed in the methyl ethyl ketone system, each spot corresponding to one of the two spots of the original diastereomeric mixture (Table I). In addition, each diastereomer gave single ninhydrin-positive spots when chromatographed in four other solvent systems (Table I). The elemental analysis values were in agreement with those calculated for β-hydroxynorleucine (Table II). Manometric Van Slyke determinations of α-carbonyl carbon with ninhydrin (9) (Table III) and of primary amino nitrogen with nitrous acid (10) (Table IV) on the products yielded values in accordance with those expected for the α-amino carboxylic acid structure.

Resolution of Optical Isomers—Resolution of the optical antipodes was accomplished by asymmetric enzymatic hydrolysis of the racemic *N*-chloroacetyl derivatives followed by acid hydrolysis of the enzyme-resistant chloroacetyl D-isomer as described by Greenstein (8). Hog renal acylase[6] was used. The *N*-chloroacetyl derivatives for the resolution were prepared from the corresponding free amino acid by acylation under the conventional Schotten–Baumann conditions [*cf.*, Greenstein and Winitz (11)]. The yields of the acyl products ranged from 70 to 90%. The melting point of the *N*-chloroacetyl racemic A was 148.0–149.5° sharp; the melting point of the *N*-chloroacetyl racemic B was 137–138° sharp.

The yield of the unacylated L-isomer thus separated was about 80% and that of the D-isomer was about 55%. The extent of hydrol-

[1] β-Hydroxynorleucine = 2-amino-3-hydroxy-1-hexanoic acid. The preparation of this compound was reported previously (4, 5).

[2] The diastereomeric forms are here arbitrarily referred to as isomer A and isomer B, the former moving faster and the latter slower when chromatographed in the methyl ethyl ketone system.

[3] Whatman cellulose powder CF-11, H. Reeve Angel & Co., Clifton, N.J.

[4] In later experiments, dry cellulose powder was packed into the column with a tightly fitting Plexiglas plunger with about the same degree of separation of the isomers.

[5] Appropriate vents for the solvent reservoir and adequate ventilation for the removal of the solvent vapors during the fractionation were provided.

[6] Nutritional Biochemical Corp., Cleveland, Ohio. Activity was determined to be 4000 μmoles of acetylmethionine hydrolyzed per hour per milligram of protein nitrogen.

Table I—Chromatographic Data of the Diastereomers of β-Hydroxynorleucine[a]

Solvent System	R_f Diastereomer A	R_f Diastereomer B
Methyl ethyl ketone system[b]	0.38–0.49 (0.43)	0.30–0.40 (0.35)
77% Ethanol	0.50–0.60 (0.55)	0.53–0.61 (0.57)
80% Pyridine	0.54–0.65 (0.58)	0.58–0.68 (0.63)
Formic acid system[c]	0.61–0.73 (0.67)	0.66–0.76 (0.71)
Phenol system[d]	0.63–0.77 (0.69)	0.63–0.77 (0.70)

[a] Chromatographed on Whatman No. 1 chromatographic paper for 18–22 hr at 22–25°, ascending. Compounds were visualized by dipping the paper into a 0.25% acetone solution of ninhydrin and then warming the paper. Figures indicate the range of the R_f values; figures in the parentheses are the R_f values at the center of ninhydrin-positive spots. [b] Described in the text. [c] Formic acid–water–2-methyl-2-propanol (15:15:70). [d] Phenol saturated with 10% sodium citrate.

Table II—Elemental Analysis Data of the Diastereomers of β-Hydroxynorleucine[a]

Diastereomer	C, % Calc.[b]	C, % Obs.	H, % Calc.	H, % Obs.	N, % Calc.	N, % Obs.
A	48.96	48.87	8.90	9.01	9.52	9.41
B	48.96	48.70	8.90	9.01	9.52	9.25

[a] The analysis was performed by the staff of the Microanalytical Laboratory, National Institute of Arthritis, Metabolic, and Digestive Diseases, National Institutes of Health, Bethesda, Md. [b] Calculated for β-hydroxynorleucine, $C_6H_{13}NO_3$.

ysis of the digest in either case was followed by the Van Slyke nitrous acid method (10). Each free amino acid antipode yielded a single spot when chromatographed on paper in the methyl ethyl ketone system. Data on the identification and purity of these products are given in Table V.

Optically pure *N*-chloroacetyl L-derivatives were prepared by acylation of the optically pure L-amino acid, as described for the acylation of the racemic mixture. Optically pure *N*-chloroacetyl D-derivatives were isolated from this resolution mixture as described by Greenstein (8). The purity of the chloroacetyl derivatives separated from the digest was determined using the Van Slyke nitrous acid method; they yielded essentially no detectable amounts of nitrogen (Table VI).

Relative Rates of Enzymatic Hydrolysis of Diastereomers—Equimolar quantities of a racemic mixture of the *N*-chloroacetyl derivatives of diastereomers A and B were treated with the same solution of hog renal acylase I, and the rates of hydrolysis of these compounds were compared (Fig. 1).

The hydrolytic rates (averages of three determinations, calculated from the initial linear portion of the curve) were as follows: diastereomer A, 125 μmoles/hr/mg of protein nitrogen; and diastereomer B, 235 μmoles/hr/mg of protein nitrogen.

Microbiological Studies—The four isomers of β-hydroxynorleucine and their corresponding *N*-chloroacetyl derivatives were screened for possible antitumor activity using *Lactobacillus casei*[7] (ATCC 7469) in riboflavin-supplemented riboflavin assay medium[8], as described by Foley *et al.* (2). The level of riboflavin added was the minimal quantity required for maximal growth [*cf.*, (3)], which was determined here by assay to be 0.03 μg of riboflavin/ml, the final concentration of the assay system. The details of the assay procedure were described previously (3).

The range of concentration of the test compound studied was 100–1000 μg/ml (final concentration). The pH of the final assay system containing the highest concentration of the test compound determined in parallel nonsterile tubes differed no more than 0.07 pH unit from the control tubes containing no test compounds. The results are summarized in Tables VII and VIII.

Table III—Van Slyke Ninhydrin Determination of α-Carbonyl Carbon of the Diastereomers of β-Hydroxynorleucine[a]

	Diastereomer A	Diastereomer B
α-Carbon taken, mg	0.407	0.407
α-Carbon found, mg	0.396	0.400
Percent theory	97.1	98.5
Percent theory, alanine control	96.8	96.8

[a] Amino acid solution (1.0 ml) containing an equivalent of 0.407 mg of α-carbon treated with 1.0 ml of saturated picric acid solution pipetted into a reaction tube and 100 mg of ninhydrin added.

Table IV—Van Slyke Nitrous Acid Determination of Primary Amino Nitrogen of Diastereomers of β-Hydroxynorleucine[a]

	Diastereomer A	Diastereomer B
Primary amino nitrogen taken, mg	0.475	0.475
Primary amino nitrogen found, mg	0.496	0.495
Percent theory	104.2	104.2
Percent theory, leucine control	101.1	99.4

[a] Amino acid solution (1.0 ml) containing an equivalent of 0.475 mg of primary amino nitrogen diluted with 4.0 ml of water and treated with 1 ml of acetic acid and 2 ml of sodium nitrite solution (80 mg/100 ml).

Table V—Purity of the Isomers of β-Hydroxynorleucine[a]

Isomer	Optical Rotation[b], $[\alpha]_D^{22-24}$ Reported[d]	Optical Rotation[b], $[\alpha]_D^{22-24}$ Observed	Nitrogen[c], % Calc.	Nitrogen[c], % Found
L-A	+18.6	+19.1	9.52	9.39
D-A	−18.5	−20.6	9.52	9.68
L-B	+27.1	+26.2	9.52	9.22
D-B	−27.4	−27.1	9.52	9.58

[a] In all five solvent systems (Table I), a single spot appeared. [b] Optical rotation was determined on a Rudolph polarimeter model 80 with a sodium lamp using 100-mm tubes (bore size 3 mm) and a sample capacity of 0.7 ml; c 2, 5 *N* HCl. [c] Micro-Kjeldahl nitrogen analysis was performed by Mr. James F. Williams of this Laboratory. Calculated for $C_6H_{13}NO_3$. [d] Reference 4.

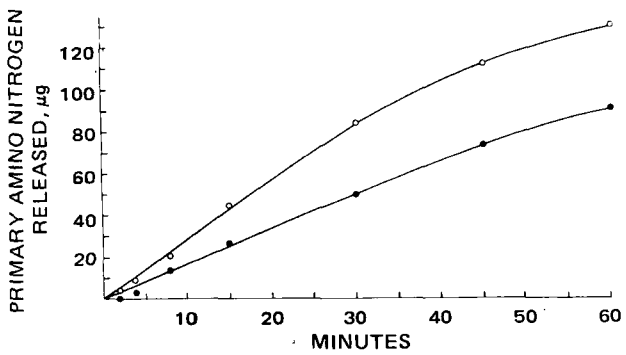


Figure 1—*Rates of hydrolysis of* N-*chloroacetyl-DL-β-hydroxynorleucine A and* N-*chloroacetyl-DL-β-hydroxynorleucine B by hog renal acylase I in 0.1* M *potassium phosphate buffer, pH 7.0,* T = *22–24°. Substrate concentration = 0.006* M *racemic mixture. Primary amino nitrogen was determined by the nitrous acid method of Van Slyke (10).*

[7] From the American Type Culture Collection, Rockville, Md.
[8] Difco Laboratories, Detroit, Mich.

Table VI—Purity of the Isomers of *N*-Chloroacetyl-β-hydroxynorleucine

Isomer[a]	Melting Point (Uncorrected)[b]	Optical Rotation[c], $[\alpha]^{21-24}$	Van Slyke Nitrous Acid Determination[d], % Primary Amino Nitrogen	C[e], %		H[e], %		N[e], %	
				Calc.	Obs.	Calc.	Obs.	Calc.	Obs.
L-A	101–104°	+27.2°	2	42.96	42.73	6.34	6.34	6.26	6.23
D-A	102–105°	−27.5°	2	42.96	43.25	6.34	6.58	6.26	6.18
L-B	144–146°	+12.0°	3	42.96	43.04	6.34	6.41	6.26	6.20
D-B	144–148°	−11.6°	0	42.96	43.20	6.34	6.50	6.26	6.32

[a] Ninhydrin reaction was negative for all isomers. Aqueous solution (concentration approximately 1 mg/ml) was spotted on paper, dried, treated with 0.25% acetone solution of ninhydrin, and warmed. [b] Melting points were determined on a Fisher–Johns melting-point block. [c] Optical rotation was determined on a Rudolph polarimeter model 80 with a sodium lamp. Conditions were as described in Table V; c 2, water. [d] Van Slyke nitrous acid determination of primary amino nitrogen, determined as described (*cf.*, Ref. 7). [e] Elemental analyses were performed by the staff of the Microanalytical Laboratory, National Institute of Arthritis, Metabolic, and Digestive Diseases, National Institutes of Health, Bethesda, Md. Calculated for $C_8H_{14}ClNO_4$.

None of the isomers of β-hydroxynorleucine as free amino acids affected the growth of *L. casei* in the system used (Table VII). However, when *N*-chloroacetylated, all four isomers showed some inhibition, with *N*-chloroacetyl-D-β-hydroxynorleucine B exhibiting about twice the growth inhibitory activity of the others (Table VIII).

DISCUSSION

The separation of the diastereomers by partition column chromatography yielded the pure isomers by a single passage of the diastereomeric mixture through the column. However, quantitative separation could not be accomplished by a single passage despite numerous attempts, varying such parameters as the geometry of the column, the particulate size of the cellulose powder, the mode of packing of the powder (solvent saturated or dry), and the flow rate.

Table VII—Effect of the Isomers of β-Hydroxynorleucine on the Growth of *L. casei* in Riboflavin Assay Medium[a]

Isomer	Turbidity[b], Klett Units (Red Filter)				Maximum Change, %
	0 μg/ml	100 μg/ml	500 μg/ml	1000 μg/ml	
L-A	188	184	187	188	0
D-A	188	184	182	180	−4
L-B	188	183	182	184	−2
D-B	188	186	184	183	−3
DL-AB	188	185	186	180	−4

[a] A typical experiment, representing five experiments. The turbidimetric figures are the averages of duplicates for that concentration. Maximum variation from mean = ±3 Klett units. [b] The instrument was set at zero against a sterile reagent blank which contained all components of the experimental tubes except the test compound and 0.05 ml of the normal saline solution that was used for washing the organism in place of the inoculum. The blank was kept in the refrigerator for the duration of the incubation of the experimental tubes. A sterility control, identical to the sterile reagent blank but incubated with the experimental tubes, was clear and read within the variation of the empty Klett tubes (±3 Klett units).

Table VIII—Effect of the Isomers of *N*-Chloroacetyl-β-hydroxynorleucine on the Growth of *L. casei* in Riboflavin Assay Medium

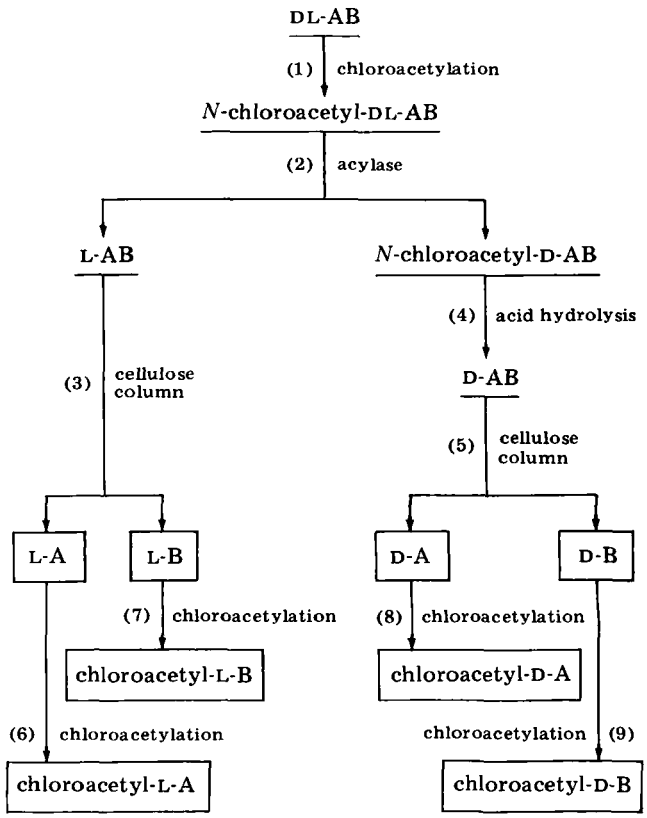
Isomer[a]	Turbidity[b], Klett Units				Maximum Change, %
	0 μg/ml	100 μg/ml	500 μg/ml	1000 μg/ml	
L-A	172	166	153	140	−19
D-A	172	162	153	134	−22
L-B	172	160	149	137	−20
D-B	172	166	129	84	−51
DL-AB	172	166	148	130	−24
Mercaptopurine	171	92	12	17	−93

[a] The pH of the test solutions was adjusted to 6 by the addition of 0.1 *N* NaOH. [b] See Table VII footnotes.

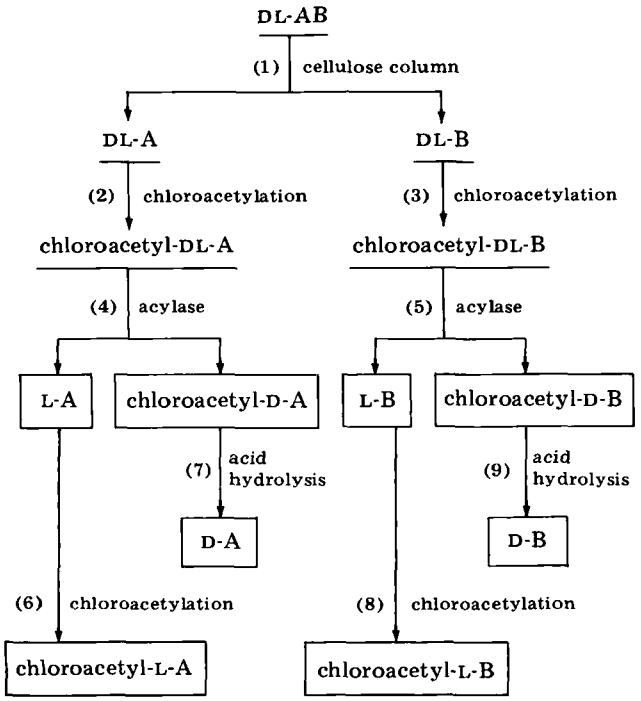
Although data are insufficient for assignment of the absolute configuration of the diastereomers, comparison of the chromatographic mobilities of these isomers in the Hardy–Holland solvent system (12) with the *threo-* and *erythro*-β-hydroxynorleucines of Buston and Bishop (5) indicates that diastereomer A, described here, corresponds to their *threo*-β-hydroxynorleucine and that diastereomer B corresponds to their *erythro*-β-hydroxynorleucine.

The four stereoisomeric forms of the free amino acid and of the *N*-chloroacetylated derivative were isolated by either Scheme I or II. The racemic diastereomers were separated by column chromatography followed by an enzymatic resolution of each racemic chloroacetyl diastereomer or by resolution of the racemic diastereomeric mixture followed by the separation of the optically homogeneous diastereomeric mixture by column chromatography.

It was noted that the optical purity of the products was retained in either route, *i.e.*, whether the separation of the diastereomers was followed by resolution or was performed on the optically ho-



Scheme I—Separation of the isomers of β-hydroxynorleucine and their N-*chloroacetyl derivatives*



Scheme II—Separation of the isomers of β-hydroxynorleucine and their N*-chloroacetyl derivatives*

mogeneous diastereomeric mixture. The selection of the scheme to be used depends upon the compound desired. Hence, Scheme I (Table IX) was preferable for the isolation of the free amino acids because only five steps were required. Scheme II was more advantageous for the isolation of all four chloroacetylated isomers because only seven steps were required by this route as compared to the nine steps needed *via* Scheme I (Table IX).

Microbial studies confirmed earlier observation (3) that although the β-hydroxyamino acids as free acid were innocuous to the growth of *L. casei* in the system used here, their chloroacetylated derivatives were moderately growth inhibitory. However, the chloroacetyl D-B-isomer was twice as active as the other three isomers.

The apparent "activation" of these amino acids by acylation appears now to be unrelated to the hydrolytic rate of the *N*-acylated derivatives by acylase; apparently, the acyl D-derivatives of neither diastereomer were substrates of this enzyme in the resolution procedure. However, that the chloroacetyl group nevertheless contributes to the activity seems likely in view of the activity exhibited by the acylated derivatives of all four isomers and the inactivity of the unacylated mother compounds. It is interesting that despite the possible availability of a chloroacetyl group and an amino acid analog, L-β-hydroxynorleucine (either diastereomer), as a result of enzymatic hydrolysis, the acyl D-B-isomer exhibited the greatest activity.

If the activity shown by the chloroacetylated racemic diastereomeric mixture is due to a single isomer, isolation of this isomer should result in a compound four times as active as the mixture. In fact, however, there was noted only a twofold increase in activity. This observed twofold increase in activity by the chloroacetylated D-B-isomer could be explained on the basis that, contrary to the assumption of dilution of activity by the inert isomers, all four

Table IX—Comparison of Routes in the Isolation of Isomers of Free and Acylated β-Hydroxynorleucine

	Scheme I		Scheme II	
Treatment	For Preparation of Free Acid	For Preparation of *N*-Acylamino Acid	For Preparation of Free Acid	For Preparation of *N*-Acyl-Amino Acid
Chloroacetylation	1[a]	5	2	4
Enzymatic hydrolysis	1	1	2	2
Column chromatography	2	2	1	1
Acid hydrolysis	1	1	2	0
Total steps	5	9	7	7

[a] The number of operations required.

chloroacetylated isomers exhibited some growth inhibitory activity. Hence, the 24% inhibition shown by the chloroacetylated racemic diastereomeric mixture is probably the resultant action of all four chloroacetylated isomers, each contributing approximately a fourth of the total activity.

In view of the observation that certain of these compounds do exhibit some growth inhibitory activity in the microbial system selected for antitumor screening, further studies against mammalian tumors are indicated. The preparation of larger quantities of the optically pure diastereomers and their respective *N*-chloroacetyl derivatives is in progress.

REFERENCES

(1) T. T. Otani and M. Winitz, *Arch. Biochem. Biophys.*, **102,** 464(1963).

(2) G. E. Foley, R. E. McCarthy, V. M. Binns, E. E. Snell, B. M. Guirard, G. W. Kidder, V. C. Dewey, and P. S. Thayer, *Ann. N.Y. Acad. Sci.*, **76,** 413(1958).

(3) T. T. Otani, *Cancer Chemother. Rep.*, **38,** 25(1964).

(4) R. T. Adams and C. Niemann, *J. Amer. Chem. Soc.*, **73,** 4260(1951).

(5) H. W. Buston and J. Bishop, *J. Biol. Chem.*, **215,** 217(1955).

(6) Y. Ikutani, T. Okuda, and S. Akabori, *Bull. Chem. Soc. Jap.*, **33,** 582(1960).

(7) T. T. Otani and M. R. Briley, *J. Pharm. Sci.*, **63,** 1253(1974).

(8) J. P. Greenstein, in "Methods in Enzymology," vol. III, S. P. Colowick and N. O. Kaplan, Eds., Academic, New York, N.Y., 1957, p. 554.

(9) D. D. Van Slyke, R. T. Dillon, D. A. MacFadyen, and P. Hamilton, *J. Biol. Chem.*, **141,** 627(1941).

(10) D. D. Van Slyke, *ibid.*, **83,** 425(1929).

(11) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," vol. III, Wiley, New York, N.Y., 1961, p. 1834.

(12) T. L. Hardy and D. O. Holland, *Chem. Ind.*, **1952,** 855.

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