para-[N-Bis-(2-chloroethyl)]-aminobenzoylglutamic Acid[1a,b]

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The L and D isomers of *p*-[N-bis-(2-chloroethyl)]-aminobenzoylglutamic acid were synthesized by two independent routes. Their identity and purity have been established by quantitative ultraviolet and infrared absorption spectra, by rotatory dispersion measurement and by neutralization titration. Both isomers, and their diethyl esters, showed inhibitory activity on five microbiological systems.

Various types of aliphatic and aromatic "nitrogen mustards" have been studied[2] and a few have been found effective as antineoplastic agents.[3] Recently introduced N-bis-(2-chloroethyl)-amino derivatives of naturally occurring amino acids, such as phenylalanine,[4] serine and threonine,[5] appear to have more selective cytotoxicity than their predecessors.

In exploring the concept of selective cytotoxicity, it was thought that a peptide, a polypeptide, or even a protein carrying an N-bis-(2-chloroethyl)-amino group could permit a specificity in interfering with a certain type of cell growth and in inhibiting some particular enzyme system(s). Interesting speculations along this line have been expressed by Danielli,[6] Ross, *et al.*,[7] Bergel, *et al.*,[8] and others.

(1) (a) Presented in part before the Medicinal Chemistry Division of the American Chemical Society, 138th Meeting, New York, September 1960; (b) this investigation was supported in part by a research grant from the National Institutes of Health, USPHS No. CY-3335.

(2) "Comparative Clinical and Biological Effects of Alkylating Agents," *Ann. N. Y. Acad. Sci.*, **68**, 657 (1958).

(3) R. B. Ross, *J. Chem. Educ.*, **26**, 368 (1959); J. A. Montgomery, *Cancer Research*, **19**, 447 (1959).

(4) F. Bergel and J. A. Stock, *J. Chem. Soc.*, 2409 (1954); L. F. Larionov, A. S. Khoklov, E. N. Shkodinskaya, O. S. Vasina, V. I. Trusheikina, and M. A. Novikova, *Lancet*, **269**, 169 (1955).

(5) F. Bergel and R. Wade, *J. Chem. Soc.*, 941 (1959).

(6) J. F. Danielli, *Nature*, **170**, 863 (1952).

(7) W. C. J. Ross, G. P. Warwick, and J. J. Roberts, *J. Chem. Soc.*, 3110 (1955).

(8) F. Bergel, J. A. Stock, and R. Wade, "Peptides and Macromolecules as Carriers of Cytotoxic Groups," in "Biological Approaches to Cancer Chemotherapy," Academic Press, New York, N. Y., 1961, p. 125.

In seeking antifolic acid agents, the possibility was contemplated of attaching an N-bis-(2-chloroethyl)-amino group to the side chain of the folic acid, *p*-aminobenzoyl-L-glutamic acid, which is a "pseudo" dipeptide. This concomitantly would offer an opportunity for studying the technique of peptide "nitrogen mustard" synthesis which apparently had not been investigated.[9] Any biological activities of *p*-[N-bis-(2-chloroethyl)]-aminobenzoyl-L-glutamic acid and related compounds would be of significance since this acid is part of a biologically active molecule and is utilized by bacteria for growth. It would also be advisable to determine whether the compound, its enantiomorph, and related intermediates, would cause any interference with the incorporation of *p*-aminobenzoic acid and *p*-aminobenzoylglutamic acid in folic acid biosynthesis.[10]

In order to obtain *p*-aminobenzoylglutamic acid and related compounds of highest purity, optically pure glutamic acid was prepared[11] for use in the synthesis. Both L and D isomers of glutamic acid were enzymatically resolved[12] and tested against L-glutamic decarboxylase and D-aminoacid oxidase.[13] The diethyl and dibenzyl esters of *p*-aminobenzoylglutamic acid[14] were synthesized by a modified acid chloride peptide condensation procedure. Other methods, such as using the mixed anhydride and carbodiimide, proved unfruitful.

By hydroxyethylation and then chlorination of the esters, the diethyl and dibenzyl N-bis-(2-chloroethyl)-aminobenzoylglutamates were obtained. However, both the crude N-bis-(2-hydroxyethyl) and N-bis-(2-chloroethyl) compounds are extremely difficult to purify and consequently the yields were poor. An alternate synthesis by condensation of N-bis-(2-chloroethyl)-aminobenzoic acid with esters of glutamic acid resulted in pure products in a desirable

(9) Since our data were presented,[18] L. F. Larionov (*Cancer Res.*, **21**, 99 (1961)) has reported the antitumor activities of some di- and tripeptides of Sarcolysin and of *p*-[N-bis-(2-chloroethyl)]-aminophenylacetic acid. However, the synthesis, optical configuration, and purity of these chemicals were not given.

(10) D. D. Wood, "Ciba Foundation Symposium, Chem. and Biol. Pteridines," Little, Brown and Co., Boston, Mass., 1954, p. 220; T. Shiota, *Arch. Biochem. and Biophys.*, **80**, 155 (1959).

(11) J. P. Greenstein, "The Resolution of Racemic α-Amino Acids," *Advances in Protein Chem.*, **9**, 121 (1954), Academic Press, Inc., New York, N. Y.

(12) The enzymatic resolution of the DL-glutamic acid was performed in the laboratory of the late Dr. J. P. Greenstein, National Cancer Institute.

(13) A. Meister, L. Levintow, R. B. Kingsley, and J. P. Greenstein, *J. Biol. Chem.*, **192**, 535 (1951).

(14) The optical rotations of the L isomers are $[\alpha]^{25}D$ −9.8° in absolute ethanol and $[\alpha]^{25}D$ +1.3° in chloroform for the diethyl and dibenzyl esters, respectively; $[\alpha]^{25}D$ −9.5° (95% ethanol) was given for diethyl *p*-aminobenzoyl-L-glutamate by D. I. Weisblat, B. J. Magerlein, and S. T. Rolfson, U. S. Patent 2,625,562 (Jan. 13, 1953).

yield. The diethyl and dibenzyl compounds formed by these two schemes are identical.

The hydrolysis of the diethyl esters to N-bis-(2-chloroethyl)-aminobenzoylglutamic acid was unfruitful: (1) by acid, using various strengths of acetic acid and hydrochloric acid, and (2) by non-aqueous alkali, using sodium methoxide in dioxane and methanol.[15] Also ending in failure was the treatment of the dibenzyl ester: (1) by hydrogenolysis with palladium catalyst, and (2) by debenzylation with saturated hydrogen bromide in glacial acetic acid.[16] Contrary to the general concept of the instability of N-bis-(2-chloroethyl)-amino compounds, both the L and D isomers of N-bis-(2-chloroethyl)-aminobenzoylglutamic acid were obtained in optically pure form by aqueous alkaline hydrolysis.[17] The compounds synthesized are listed in Table I.

The L and D isomers of p-[N-bis-(2-chloroethyl)]-aminobenzoylglutamic acid, as well as their esters, are waxy crystals with wide melting range. However, their identity and purity can be established: (1) by identical ultraviolet and infrared spectra, and (2) by optical rotation of equal magnitude but opposite sign for the corresponding enantiomorphs. The rotatory dispersion curves of L- and D-p-[N-bis-(2-chloroethyl)]-aminobenzoylglutamic acid, shown in Fig. 1 (an anomalous dispersion), demonstrated further that they are pure antipodes, although very low optical rotation was observed at 589 mμ (sodium D-line). The p-[N-bis-(2-chloroethyl)]-aminobenzoylglutamic acid and its diethyl ester give very similar infrared absorption spectra as shown in Table II. By neutralization titration, correct values for neutralization were obtained.

Preliminary data on the biological activity of this group of "nitrogen mustards" indicate moderate inhibition,[18] ID_{50} = 10 to 100 μg./ml., in these microbiological assay systems: (a) *Streptococcus faecalis*–PGA; (b) *Lactobacillus fermenti*–thiamine; (c) *Lactobacillus casei*–riboflavin; (d) *Escherichia coli* in inorganic salts and glucose medium, and (e) *Candida albicans* in a synthetic medium in which

(15) G. D. Fasman and E. R. Blout, *J. Am. Chem. Soc.*, **82**, 2262 (1960).

(16) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).

(17) J. L. Everett, *et al.*,[21] have reported that p-[N-bis-(2-chloroethyl)]-aminobenzoic acid and its ethyl ester liberated 1% chlorine in 50% acetone at 66° in 30 min. However, ethyl p-[N-bis-(2-chloroethyl)]-aminobenzoate cannot be hydrolyzed to the free acid under the given aqueous alkaline condition.

(18) Studied by Dr. G. E. Foley, *et al.*, of this Laboratory.

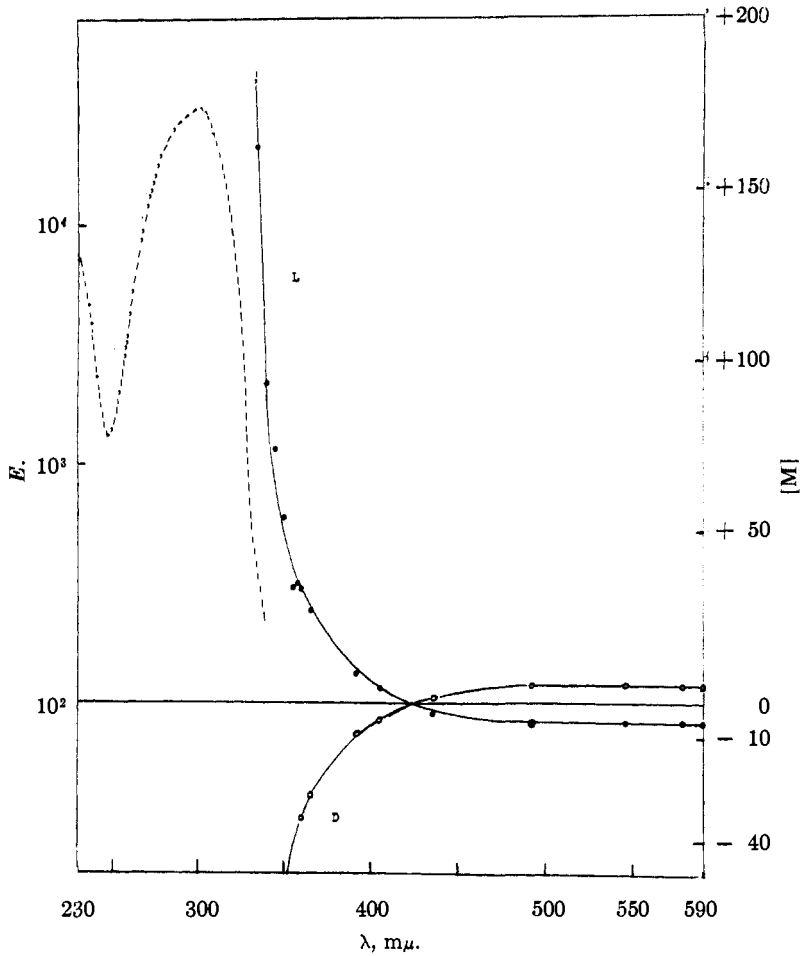
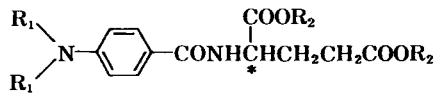


Fig. 1.—Ultraviolet absorption and rotatory dispersion of L-and D-*p*-[N-bis-(2-chloroethyl)]-aminobenzoylglutamic acid.

thiamine was the limiting growth factor. Identical activity was observed for L and D isomers.[19]

(19) The antineoplastic activities are at present under investigation.

TABLE I



	R_1	R_2	Optical config.	$[\alpha]^{25}D$ Abs. ethanol	$[\alpha]^{25}D$ Chloroform	Formula	Calcd., % C	Calcd., % H	Calcd., % N	Calcd., % Cl	Found, % C	Found, % H	Found, % N	Found, % Cl
I	$HOCH_2CH_2$	C_2H_5	L			$C_{20}H_{30}N_2O_7$	58.5	7.4	6.8		58.3	7.8	7.0	
II	$HOCH_2CH_2$	$C_6H_5CH_2$	L			$C_{30}H_{34}N_2O_7$	67.4	6.4	5.2		66.9	6.3	5.1	
III	$ClCH_2CH_2$	C_2H_5	L	−8.1°	+12.6°	$C_{20}H_{28}N_2Cl_2O_5$	53.7	6.3	6.3	15.9	53.8	6.1	6.8	16.0
IV	$ClCH_2CH_2$	C_2H_5	D	+8.2°	−12.3°	$C_{20}H_{28}N_2Cl_2O_5$	53.7	6.3	6.3	15.9	54.2	6.3	6.4	16.0
V	$ClCH_2CH_2$	$C_6H_5CH_2$	L	Insol.	− 1.6°	$C_{30}H_{32}N_2Cl_2O_5$	63.1	5.7	4.9	12.4	63.1	5.5	4.9	12.7
VI	$ClCH_2CH_2$	H	L	−1.3°	Insol.	$C_{16}H_{20}N_2Cl_2O_5$	49.1	5.2	7.2	18.1	49.2	5.5	7.0	17.8
VII	$ClCH_2CH_2$	H	D	+1.3°	Insol.	$C_{16}H_{20}N_2Cl_2O_5$	49.1	5.2	7.2	18.1	49.6	5.3	7.0	18.2

TABLE II[19a]: INFRARED ABSORPTION SPECTRA, IN CM.$^{-1}$ OF p-[N-BIS-(2-CHLOROETHYL)]AMINOBENZOYLGLUTAMIC ACID AND ITS DIETHYL ESTER (1% SOLUTION IN CHLOROFORM)

Acid		Ester	
1740 (s)	C=O acid	1725 (s)	C=O ester
1660 (s)	C=O amide (Amide I)	1650 (s)	C=O amide (Amide I)
1610 (s)	C=C aromatic ring	1610 (s)	C=C aromatic ring
1530 (w)	N—H and C—N amide (Amide II)	1528 (w)	N—H and C—N amide (Amide II)
1503 (s)	C=C aromatic ring	1500 (s)	C=C aromatic ring
		1448 (m)	
1395 (m) 1211 (s)			
1186 (s)	C—N amide (Amide III)	1185 (s)	C—N amide (Amide III)
		1097 (m)	
		1040 (m)	
829 (m) 765 (m)		830 (m)	

(19a) In assigning the absorption bands, acknowledgment is given to Drs. C. deLozé and E. R. Blout of this laboratory.

Experimental[20]

Preparation of Nitrogen Mustards

A. Hydroxyethylation and Chlorination of Diethyl and Dibenzyl *p*-Aminobenzoylglutamates. 1. **N-Carbobenzoxy-*p*-aminobenzoic Acid.**—Twice recrystallized *p*-aminobenzoic acid was treated with a slight excess of carbobenzoxy chloride in alkaline solution by the usual Schotten–Baumann procedure. The crude compound precipitated when the reaction mixture was acidified with concd. hydrochloric acid to pH 2. The crude sample was recrystallized from glacial acetic acid, or ethyl acetate and precipitated by hexane; yield 85%; m.p. 217° dec.

Anal. Calcd. for $C_{15}H_{13}NO_4$: C, 66.4; H, 4.8; N, 5.2. Found: C, 66.4; H, 5.1; N, 4.8.

2. **Diethyl N-Carbobenzoxy-*p*-aminobenzoylglutamate.**—N-Carbobenzoxy-*p*-aminobenzoic acid (5 mM.) was suspended in 30 ml. of dry ether and phosphorus pentachloride (5.5 mM.) was added. The reaction mixture was stirred vigorously at 0–5° for 2 hr., during which time the phosphorus pentachloride disappeared completely. The acid chloride suspension in ether, after washing with ice-cooled water, was poured into a precooled solution of diethyl glutamate hydrochloride (5 mM.) and sodium hydrogen carbonate (50 mM.) in a mixture of 20 ml. of ethyl acetate and 20 ml. of water. Evolution of carbon dioxide commenced immediately and stirring was continued for 30 minutes at 0–5° and then for 1 hr. at room temperature. The organic layer was separated and washed consecutively with (1) 10 ml. of water, (2) 10 ml. of *N* hydrochloric acid, and again, (3) 10 ml. of water. After the solution was dried over anhydrous sodium sulfate and evaporated under reduced pressure, a thick syrup was obtained. By treating the syrup with hexane at 0°, a white precipitate was formed. The crude product was dissolved in absolute ethanol and filtered through Darco. When the clear filtrate was heated to boiling and diluted with water until cloudy, a pure product was obtained after cooling overnight at 4°; yield (L isomer) 56%; m.p. 128° dec.

Anal. Calcd. for $C_{24}H_{28}N_2O_7$: C, 63.1; H, 6.2; N, 6.1. Found: C, 63.8; H, 6.0; N, 6.0.

3. **Dibenzyl N-Carbobenzoxy-*p*-aminobenzoylglutamate.**—Dibenzyl glutamate was used for the condensation instead of the diethyl ester, according to the procedure in (2); yield (L isomer) 60%; m.p. 139–140°.

Anal. Calcd. for $C_{34}H_{32}N_2O_7$: C, 70.3; H, 5.6; N, 4.8; Found: C, 69.9; H, 5.4; N, 4.9.

4. **Diethyl *p*-Aminobenzoylglutamate.**—Diethyl N-carbobenzoxy-*p*-aminobenzoyl-L-glutamate (1 mM.) was decarbobenzoxylated in 10 ml. of 40% hydrogen bromide in glacial acetic acid at 25° for 30 minutes.[15] Only slight precipitation was observed. The ethereal solution was evaporated to dryness under reduced pressure. The sirup was dissolved in absolute ethanol, neutralized with

(20) All melting points are corrected and, unless otherwise specified, the compounds always become clear liquid at the melting range. The yield given is the purified compound obtained as the result of series runs of the same procedure. The elementary analyses were performed by Dr. C. K. Fitz, Box 115, Needham Heights 94, Mass., and a portion of the nitrogen determinations were made by Mrs. E. Passweg of this laboratory.

pyridine, filtered through Darco and finally precipitated with twice its volume of water; yield (L isomer) 70%; m.p. 140–141°.[14]

Anal. Calcd. for $C_{16}H_{22}N_2O_5$: C, 59.6; H, 6.9; N, 8.7. Found: C, 60.0; H, 6.8, N, 8.8.

5. **Dibenzyl p-Aminobenzoylglutamate.**—The ester was prepared as described for the ethyl ester (4); yield (L isomer) 70%; m.p. 140–141°.

Anal. Calcd. for $C_{26}H_{26}N_2O_5$: C, 69.9; H, 5.9; N, 6.3. Found: C, 69.7; H, 5.7; N, 6.2.

6. **Diethyl p-[N-Bis-(2-hydroxyethyl)]-aminobenzoylglutamate.**—Diethyl p-aminobenzoylglutamate (3 mM.) was suspended in 20 ml. of 45% acetic acid and chilled to 4°. Ethylene oxide (4 ml.) was added with vigorous stirring, first at 4° for 30 minutes, and then at room temperature for 24 hr. The clear yellowish solution was poured into 100 ml. of water and a slight excess of sodium hydrogen carbonate was added. The gummy precipitate was extracted into ethyl acetate and dried over anhydrous magnesium sulfate. After removal of the solvent, an opaque oil was obtained. In order to remove the last trace of water, the thick oil was evaporated three times with 5 ml. portions of benzene. The oil can be purified by repeatedly dissolving it in ethyl acetate, treating with Darco, and finally precipitating with hexane: yield (L isomer) 32%; an oil.

7. **Dibenzyl p-[N-Bis-(2-hydroxyethyl)]-aminobenzoylglutamate.**—The hydroxyethylation of dibenzyl p-aminobenzoylglutamate was performed as described in (6); yield (L isomer) 20%; a semi-solid.

8. **Diethyl p-[N-Bis-(2-chloroethyl)]-aminobenzoylglutamate.**—Diethyl p-[N-bis-(2-hydroxyethyl)]-aminobenzoylglutamate (5 mM.) was dissolved in 20 ml. of dry benzene and 2.5 molar equivalents of thionyl chloride was added slowly through the top of a condenser. A vigorous reaction took place immediately. After it subsided, the mixture was heated under reflux for 30 min., protected from moisture. An oil was obtained when the solvent and the excess thionyl chloride were removed under reduced pressure. The oil was dissolved in ethyl acetate and was washed consecutively with (1) twice 10 ml. of 0.5 *N* sodium hydroxide; (2) 10 ml. of water, and (3) 10 ml. of 2 *N* hydrochloric acid. The solution was then passed through a 10 × 200 mm. column packed with 160-mesh alumina and then a Darco column of the same size. After evaporating the ethyl acetate, a pale yellow semi-solid resulted which was purified by dissolving in 5 ml. of chloroform and then treating with hexane. A waxy white precipitate was formed after 4 days standing at 4°; yield (L and D isomers) 4–12%; m.p. 66–68° (90° clear).

9. **Dibenzyl p-[N-Bis-(2-chloroethyl)]-aminobenzoylglutamate.**—Chlorination of dibenzyl p-[N-bis-(2-hydroxyethyl)]-aminobenzoylglutamate was performed as described in (8); yield (L isomer) 4–12%; m.p. 92–96° (100° clear).

B. **Condensation of p-[N-Bis-(2-chloroethyl)]-aminobenzoic Acid with Diethyl and Dibenzyl Glutamate.**—Repeated trials of the synthetic procedure given by Everett, *et al.*,[21] for p-[N-bis-(2-chloroethyl)]-aminobenzoic acid afforded too small a yield, and a modified procedure was introduced. Purified methyl or ethyl p-aminobenzoate (30 mM.) was hydroxyethylated with ethylene oxide (20 ml.) in

(21) J. L. Everett, J. J. Roberts, and W. C. J. Ross, *J. Chem. Soc.*, 2386 (1953).

40% acetic acid for 24 hr. at room temperature. The isolation procedure was that described in (A-8). The methyl or ethyl *p*-[N-bis-(2-hydroxyethyl)]-aminobenzoate was recrystallized from benzene; yield 50 and 79%; m.p. 116–117° and 88–90°,[22] respectively.

Anal. Methyl *p*-[N-bis-(2-hydroxyethyl)]-aminobenzoate; calcd. for $C_{12}H_{17}NO_4$: C, 60.2; H, 7.2; N, 5.9. Found: C, 60.5; H, 7.1; N, 5.9.

The chlorination was accomplished by dissolving the bis-hydroxyethyl compound in benzene and then heating under reflux with 2.5 molar equivalents of thionyl chloride for 30 min. The *p*-[N-bis-(2-chloroethyl)]-aminobenzoate esters were isolated after evaporating the solvent and the excess of thionyl chloride, and then recrystallized from hexane; yield 75 and 75%; m.p. 63°[22] and 60–60.5°[22], respectively, for the methyl and ethyl ester. The hydrolysis of the N-bis-chloroethyl esters was performed by refluxing with 3 to 5 molar equivalents of concentrated hydrochloric acid for 20 minutes; yield 70%; m.p. 169°.[23]

1. The *p*-[N-bis-(2-chloroethyl)]-aminobenzoic acid (5 mM.) was suspended in 15 ml. of dry ether and chilled to 0–5° with ice. Phosphorus pentachloride (5.5 mM.) was added, and the reaction mixture was stirred vigorously for 1 hr. Since the acid chloride formed was only partly soluble, the suspension of acid chloride was poured with vigorous stirring into a precooled mixture of glutamic acid diethyl ester hydrochloride (5 mM.) and sodium hydrogen carbonate (50 mM.) in 20 ml. of ethyl acetate and 20 ml. of water. As the reaction proceeded, carbon dioxide was evolved. As soon as the foaming was over, stirring was continued at room temperature for 1 hr. The organic layer was separated, washed with (1) twice, 10 ml. of 0.5 *N* sodium hydroxide, (2) 10 ml. of water, and (3) 10 ml. of 2 *N* hydrochloric acid. The washed organic layer was dried over anhydrous sodium sulfate. A thick oil was obtained after evaporating the solvent, and it became a white, powdery precipitate upon standing under hexane for 4 days at 4°. Purification was accomplished by dissolving in chloroform and precipitating it with hexane; yield (L and D isomers) 80%; m.p. 66–68° (90° clear). The compound gives correct analyses and identical infrared and ultraviolet spectra as given for A-8.

2. When dibenzyl glutamate was used instead of the diethyl ester, dibenzyl *p*-[N-bis-(2-chloroethyl)]-aminobenzoylglutamate was obtained according to the procedure in (1); yield (L isomer) 82%; m.p. 92–96° (100° clear).

C. **Alkaline Hydrolysis of Diethyl *p*-[N-Bis-(2-chloroethyl)]-aminobenzoylglutamate.**—Diethyl *p*-[N-bis-(2-chloroethyl)]-aminobenzoylglutamate (6 mM.) from either reaction (A-8) or (B-1) was suspended in 11 ml. of absolute ethanol and chilled to 0–5°. Sodium hydroxide solution of *N* strength (11 ml.) was added with vigorous stirring, which was continued for 10 min., after which time the hydrolysis was allowed to proceed further at 25–30° for an additional 85 min. A clear solution resulted; by acidifying the solution with concentrated hydrochloric acid at 0° to pH 3, an opaque oil precipitated. The oil obtained after removal of the solvent was purified by dissolving in chloroform, filtering through Darco, and precipitating with hexane. The purified oil became a solid by triturating in Dry-Ice–acetone cooling mixture. It was a white powder at −30°,

(22) J. L. Everett and W. C. J. Ross, *J. Chem. Soc.*, 1972 (1949).
(23) W. C. J. Ross, *ibid.*, 183 (1949).

but turned to an oil again when brought back to room temperature. The oil was dried over phosphorus pentoxide in a vacuum desiccator and again became a solid which was very hygroscopic; yield (L and D isomers) 70%; m.p. 92° (100° clear, in sealed capillary).

Physical Measurements

A. Absorption Spectra.—A Beckman DU spectrophotometer was used for the ultraviolet absorption spectra and absolute ethanol was chosen as the solvent. A Perkin-Elmer Model 21 infrared spectrophotometer was used for the infrared absorption spectra measurements, and chloroform as the solvent.

B. Optical Rotation.—A Bellingham and Stanley polarimeter was used for the optical rotation at sodium D line, and a Rudolf photoelectric polarimeter, Model 200, with high pressure mercury lamp, for the rotatory dispersion measurements.

C. Neutralization Titration.—A Beckman Model GS pH meter and an E. Greiner ultramicroburet of 1 ml. capacity were used. Since the *p*-[N-bis-(2-chloroethyl)]-aminobenzoylglutamic acid was not soluble in 10% ethanol, the compound, about 20 mg., was dissolved by addition of 1.5 equivalents of standardized *N* sodium hydroxide. Standardized *N* hydrochloric acid was used for the back titration. The neutralization constants are 780 and 784 for the L- and D-*p*-[N-bis-(2-chloroethyl)]-aminobenzoylglutamic acid, respectively, against the calculated value of 782.6.

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