Amino Acid Composition of Upland Cotton Squares and Arizona Wild Cotton Bolls

MARCUS L. BURKS and NORMAN W. EARLE

Entomology Research Division, Agricultural Research Service, U. S. Department of Agriculture, Baton Rouge, La.

Protein and free amino acids from two sizes of cotton flower buds were analyzed by ion exchange chromatography to provide information to be used in the development of artificial diets for the boll weevil (*Anthonomus grandis* Boheman). Amino acids from several parts of the bud were analyzed; the most complete data were obtained from the anthers, since these constitute the adult weevil's main source of food. Only 9.4% of the total amino acids in the anthers occurred in the free form; of these, asparagine, glutamine, and proline were present in the greatest amounts. Amino acids in the protein were present in amounts comparable to those in other plant proteins, with relatively large quantities of aspartic and glutamic acids, leucine, alanine, glycine, and lysine.

Artificial diets are used at different laboratories for rearing the boll weevil, *Anthonomus grandis* Boheman (*1*, *3*, *10–12*). However, for certain research purposes, these diets are unsuitable, primarily because they differ in composition from the cotton flower bud (commonly called the square) on which the weevil feeds. For example, diets containing casein would not be expected to have the same relative amounts of amino acids as the cotton plant.

Diets closely resembling the weevil's natural food were needed for studies of the physiology of diapause, host plant resistance, and fatty acid and carbohydrate metabolism. To develop a satisfactory laboratory diet, a knowledge of the composition of the insect's natural food was needed. Amino acids were considered first, since these are among the most important dietary constituents. A survey of the literature revealed numerous reports on analyses of cottonseed and its by-products but almost no information on amino acids in cotton squares. Therefore, analytical work was undertaken to develop the desired information.

Selection of plant parts for analysis was based on the feeding habits of the weevil. The adult weevil perforates the outer square covering (sepals and petals) and feeds on immature anthers. The female lays its eggs individually among the anthers in cavities formed by feeding. Newly hatched larvae feed on the anthers for several days and then migrate to the ovarial region in the center of the cotton square.

The amino acids in the anthers were considered to be of greatest interest; thus, most analyses involved this part of the flower bud. A few samples of sepals and petals and of ovaries were analyzed for comparison. The amino acid composition of the natural food of the closely related thurberia weevil, *Anthonomus grandis thurberiae* Pierce, was also determined. For this purpose, a sample of young bolls from the host plant of this species was analyzed.

Experimental

Selection and Preparation of Samples. An American Upland cotton, D.P.L.–15 (*Gossypium hirsutum* L.) used in many cotton-growing areas, was the source of squares. With bracteoles and stems removed, squares were divided into two groups according to size; the smaller squares weighed 0.3 ± 0.1 gram and the larger, 0.6 ± 0.1 gram. Adult weevils feed readily on both sizes, but lay most of their eggs in the smaller (*4*).

Squares of each of the two sizes, collected at two different times during the year, were analyzed for anther amino acids. The squares collected in February came from the greenhouse and those collected in June from plants in the field. A third collection of the smaller squares, taken from the field in December, was analyzed for anther, sepal, and ovary amino acids.

A sample of bolls from the thurberia weevil host cotton, *Gossypium thurberi* Todaro, was obtained in February from greenhouse plants. The total weight of seven bolls, with bracteoles and stems removed, was 5.57 grams.

In preparing samples of squares for extraction, the sepals and petals (*A*, Figure 1) were removed after cutting a ring around the middle of the square with a razor blade, at point *D*. The anthers, *B*, which comprised the sample of greatest interest, were then scraped from the style with jewelers' forceps. The ovary and style, *C*, were removed by making a transverse cut along line *E* near the base of the square. During dissection, the samples were cooled with crushed ice. Sufficient absolute ethanol was added to make a final concentration of 80% ethanol by volume. Samples not immediately extracted were stored at −15° C.

For extraction of free amino acids, tissues were homogenized for 2 minutes in a VirTis 45 homogenizer, then transferred to a tube for centrifugation, after which the supernatant was decanted. The residues were extracted three additional times with 80% ethanol. The extracts were combined and brought to dryness on a rotary evaporator, the temperature not exceeding 50° C. The amino acids were then dissolved in sodium citrate buffer (pH 3.25) and stored at −15° C.

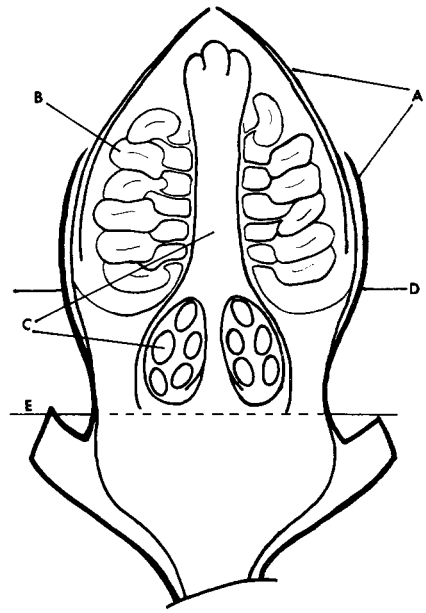

Figure 1. Cotton square showing selected parts

A. Sepals and petals
B. Anthers
C. Style and ovaries
D. Point at which sepals and petals were removed
E. Point at which ovaries were removed from base

Bolls of *G. thurberi* were diced and the free amino acids extracted, dissolved in buffer, and stored as described above.

Since the chromatographic procedure will not separate asparagine and glutamine from threonine and serine, they were converted to aspartic and glutamic acids by refluxing a small portion of the

ethanol extract with an equal volume of constant boiling hydrochloric acid for 1 hour. The differences between the quantities of aspartic and glutamic acids found before and after mild hydrolysis represented the amounts of asparagine and glutamine present. The conversion of asparagine and glutamine to their respective acids also made it possible to determine threonine and serine.

To determine to what extent bound amino acids were extracted from anthers by 80% ethanol, the amino acids in an extract of anthers were determined before and after an 18-hour acid hydrolysis. Increases in amino acids resulting from hydrolysis were attributed to peptides or other bound amino acids in the 80% ethanol extract.

The residues remaining after the extraction of free amino acids were further extracted with absolute ethanol and petroleum ether, dried in a desiccator over calcium chloride, then hydrolyzed in constant boiling hydrochloric acid distilled from an all-glass apparatus. A 50-mg. portion of each residue was refluxed in 50 ml. of acid for 18 hours. As recommended by Dustin (*2*), a relatively large volume of acid was used for samples containing large amounts of carbohydrates. The hydrochloric acid was removed in a vacuum desiccator containing sodium hydroxide, and the amino acids were dissolved in pH 3.25 buffer. Insoluble materials were removed by filtration. The filtrates were stored until use at −15° C.

Analysis. The ion exchange column chromatography procedure described by Moore, Spackman, and Stein (*7*) was used to identify the amino acids. Two-milliliter effluent fractions were collected with siphon-operated automatic fraction collectors and the fractions were heated in an electric tube heater with a ninhydrin reagent (*8*). Absorbance was measured with a Spectronic 20 colorimeter. Results were expressed as micromoles per gram wet weight of sample.

An automatic stopcock changer was designed and built to change buffer solutions on the long column after about 20 hours' operation. Two buffer-containing flasks under equal pressure were attached to a three-way stopcock above the column. A small low-speed motor, mounted in position to rotate the stopcock, was started at a predetermined time with an electric timer and stopped by a cam-operated switch after one-half revolution. This procedure allowed more continuous operation of the columns, and permitted the completion of three analyses in 1 week.

Mixtures made up of known amounts of 16 different amino acids were chromatographed four or more times to determine per cent recoveries. The standard deviation for all analyses averaged ±9.8%. Four of the amino acids had standard deviations greater than the average: aspartic acid, proline, glycine, and alanine. The per cent recoveries of ammonia and the methionine sulfoxides were not determined; values for these constituents were reported as leucine equivalents.

Table I. Amino Acids in Anthers of Cotton Squares of Different Sizes

(μmoles amino acid per gram wet weight)

	Free Amino Acids[a]		Protein Amino Acids[a]		Peptide Amino Acids,[b]
	Small	Large	Small	Large	Mixed
Aspartic acid	0.71	0.53	31.6	31.6	0.72
Asparagine	12.50	7.27	[c]	[c]	[c]
Threonine	0.71	0.63	15.2	16.3	0.24
Serine	1.52	1.29	22.0	21.9	0.72
Glutamine	6.32	4.59	[c]	[c]	[c]
Glutamic acid	2.39	2.12	38.4	37.1	1.82
Proline	2.01	10.76	19.1	17.2	0.59
Glycine	0.74	0.46	28.9	30.6	1.13
Alanine	2.80	2.20	28.4	27.9	0.85
Valine	0.95	0.61	21.6	20.5	0.42
Methionine	0.31[d]	0.24[d]	5.6	5.9	0.23
Isoleucine	0.55	0.37	18.1	18.8	0.58
Leucine	0.90	0.63	27.9	26.9	0.62
Tyrosine	0.10	0.23	9.4	8.5	0.20
Phenylalanine	0.12	0.25	12.0	12.4	0.30
Peak *B*[e]	[f]	[f]	0.7	0.7	0.36
Peak *C*[e]	0.12	0.14	[f]	[f]	[f]
Lysine	0.56	0.56	26.3	26.8	0.41
Histidine	0.30	0.44	6.8	6.9	0.04
Ammonia[e]	6.33	6.11	34.6	33.3	3.21
Arginine	0.59	0.33	14.8	13.5	0.11

[a] Average values for samples collected in February and June. [b] Extracted by 80% ethanol. [c] Amide included in respective amino acid value. [d] Includes methionine sulfoxides. [e] Reported as leucine equivalents. [f] Below sensitivity of method.

Table II. Amino Acids of Three Cotton Square Parts Collected from Field in December

(μmoles amino acid per gram wet weight)

	Free Amino Acids			Protein Amino Acids		
Amino acid	Anthers	Ovaries	Sepals	Anthers	Ovaries	Sepals
Aspartic acid	3.45	1.07	0.77	26.3	29.8	22.0
Asparagine	36.20	24.15	9.94	[a]	[a]	[a]
Threonine	1.28	0.39	0.37	13.1	14.4	12.3
Serine	2.93	1.75	1.64	17.8	19.9	16.0
Glutamine	7.52	2.49	1.78	[a]	[a]	[a]
Glutamic acid	2.71	3.16	1.39	28.8	37.6	27.9
Proline	Trace	Trace	Trace	14.7	22.7	15.7
Glycine	0.39	0.51	0.11	25.4	29.6	23.2
Alanine	3.00	2.46	1.48	24.5	28.6	20.2
Valine	1.51	0.36	0.15	18.0	20.1	14.0
Methionine	0.23	Trace	[b]	4.1	6.1	1.2
Isoleucine	1.63	0.19	0.11	14.7	17.5	12.4
Leucine	1.21	0.23	0.09	22.7	26.9	18.6
Tyrosine	0.22	[b]	[b]	7.4	5.9	5.5
Phenylalanine	0.25	[b]	[b]	9.0	11.0	6.1
Peak *A*[c]	[b]	1.50	[b]	[b]	[b]	[b]
Peak *B*[c]	[b]	[b]	0.08	[b]	0.5	[b]
Peak *C*[c]	1.11	0.61	0.25	[b]	[b]	[b]
Lysine	0.67	0.17	0.16	21.5	30.0	20.6
Histidine	2.64	0.95	0.57	5.7	7.1	4.9
Ammonia[c]	3.19	3.28	3.25[d]	32.1	35.1	25.7
Peak *D*[c]	[b]	[b]	0.17	[b]	[b]	[b]
Arginine	0.62	3.02	0.09	13.2	18.4	10.1

[a] Amide included in respective amino acid value. [b] Below sensitivity of method. [c] Reported as leucine equivalents. [d] Two peaks reported as ammonia.

The recoveries of asparagine and glutamine as aspartic and glutamic acids were found to be 100%. Analyses of cystine and tryptophan in protein were not attempted.

The total nitrogen for free amino acid extracts and protein hydrolyzates was determined by a ninhydrin procedure adapted from the procedures described by Fels and Veatch (*5*) and Kanchakk (*6*).

Results

Free Amino Acids. Twenty components were detected in the alcoholic extracts of anthers, as shown in Table I. The data as shown are averages of the samples taken in February from the greenhouse and in June from the field. The two major components in decreasing order for small squares were asparagine and glutamine, and for the large squares were proline and asparagine. Asparagine, glutamine, proline, and ammonia comprised about 70% of the total ninhydrin-positive materials identified.

Only serine, glutamic acid, and alanine in addition to the three components mentioned above, were found in quantities greater than 1 μmole per gram wet weight.

A component occurring only in the extracts of February greenhouse collections of *hirsutum* anthers and *thurberi* bolls appeared in the effluent slightly before aspartic acid. This component, thought to be methionine sulfoxide, was reported as methionine. In each case methionine was very low or absent.

An unidentified material (peak *C*) occurred in all free amino acid extracts. Its position was identical to that of hydroxylysine; however, it was destroyed by mild hydrolysis and was not believed to be hydroxylysine. An unknown material also occurred in hydrolyzates of protein and free amino acid samples (peak *B*).

In Table II, a complete amino acid analysis of small squares collected in December is shown. Except for glutamic acid, glycine, and arginine, each free amino acid decreased in quantity from anthers to ovaries to sepals. The largest free component in each type of tissue was asparagine, which comprised 51% of the total in anthers, 52% in ovaries, and 44% in sepals. An unidentified material (peak *A*) occurred in the ovary analysis in a position similar to that of γ-aminobutyric acid. The identification of this component was not confirmed. Plaisted (*9*), however, identified γ-aminobutyric acid among the free amino acids from the leaves of cotton.

Generally, the values for anther free amino acids in the February and June collections were less than those in the December collection. The total, for large or small squares, in February and June was less than 60% of that in December. Total amino acids recovered from the ovary extract were about one third less than those from the anthers, and those from the sepals about two thirds less.

The unidentified components (peaks *B* and *D*) in the sepal extract were not found in other free amino acid extracts. Peak *B*, however, appeared to be similar to unidentified peaks in several of the hydrolyzates. A component of the sepal extract, reported as ammonia, appeared to consist of two components; the second was not identified.

The amount of asparagine (as shown in Table III) in the bolls of *G. thurberi* was about twice as large as the total of all other free amino acids. The second largest, glutamine, occurred in quantities similar to those found in square anthers. Except for aspartic acid and threonine, most free amino acids were detected in smaller quantities in *thurberi* bolls than in *hirsutum* squares.

Peptide Amino Acids. The sample used to determine that bound amino acids were being extracted by 80% ethanol was of mixed sizes. The increases (shown in Table I) after hydrolysis of the extract indicated that significant amounts of peptide or otherwise bound amino acids were being extracted.

Protein Amino Acids. The amino acids as detected in the hydrolyzed protein samples from squares are also shown in Tables I and II. Eighteen components were found in the anthers, one of which (peak *B*) was not identified.

The results shown in Table I are an average of the data from February greenhouse and June field square anthers. The quantities from the two sizes were similar. The constituents of the ovary and sepal proteins from the December collection (Table II) were similar to anther protein; however, they varied a great deal in total amount. The ovary total was 22% greater and the sepal total 14% less than in anthers of the same squares.

Thurberi boll protein constituents, as shown in Table III, were very similar to those found in *hirsutum* square anthers. The total amount of protein in the bolls, however, was only 50% of that from anthers.

The largest constituents in the anther and boll samples were aspartic and glutamic acids, glycine, and alanine. The presence of large amounts of ammonia in the hydrolyzates indicated that part of the aspartic and glutamic acids present might have arisen from the related amino acids asparagine and glutamine.

Table III. Amino Acids of Thurberi Bolls

(μmoles amino acid per gram wet weight)

Amino Acid	Free Amino Acids	Protein Amino Acids
Aspartic acid	1.17	20.7
Asparagine	40.90	[a]
Threonine	1.20	7.9
Serine	1.74	10.6
Glutamine	5.36	[a]
Glutamic acid	1.66	20.1
Proline	1.61	8.8
Glycine	0.18	14.5
Alanine	2.13	12.4
Valine	0.50	11.2
Methionine	0.08[b]	2.3
Isoleucine	0.12	8.3
Leucine	0.05	13.2
Tyrosine	[c]	4.3
Phenylalanine	[c]	6.0
Peak *C*[d]	0.18	[c]
Lysine	0.34	12.3
Histidine	0.43	3.4
Ammonia[d]	2.55	26.1
Arginine	0.36	9.5

[a] Amide included in respective amino acid value. [b] Includes methionine sulfoxides. [c] Below sensitivity of method. [d] Reported as leucine equivalents.

Discussion

Collections of cotton squares analyzed from the greenhouse in February and the field in June and December (equivalent to late fall) were considered a cross section of plant growth conditions. Individual differences did occur between the February and June collections, but these were less pronounced than differences due to square size.

The results of analyses of cotton anthers differed in several respects from those reported by Plaisted (*9*) for cotton leaves. The ratio of free to protein amino acids in leaves was about twice that in anthers. The leaves also contained greater amounts of free aspartic and glutamic acids. The relative amounts of the various amino acids in anther and leaf protein were also dissimilar.

Of the 23 ninhydrin-positive components detected in the various samples of cotton squares, four remained unidentified. The only one of these found in large enough amounts to be of interest was suspected to be γ-aminobutyric acid.

The total nitrogen values for the 80% ethanol (free amino acid) extracts ranged from 1.89 mg. per gram wet weight for large anthers collected in June to 3.49 mg. for anthers collected in December. The free and peptide amino acids accounted for only 37 to 69% of the total nitrogen in these extracts. About one fourth of this was in the form of peptides.

The values for total nitrogen in the protein hydrolyzates ranged from 5.6 mg. per gram wet weight for anthers collected in December to 8.6 mg. for ovaries collected in December. Recovery of nitrogen as amino acids from anther protein based on total nitrogen was 98.5 to 106.2%. The recoveries for ovary and sepal samples were 86 and 82%, respectively.

The applicability of these data to nutritional research is currently being investigated. Preliminary results with artificial diets have been very promising. A diet for adult weevils containing an acetone powder of cotton squares as the protein source, and a mixture of L-amino acids based on analyses of anthers, has given very good results. A laboratory culture of weevils maintained an average oviposition rate of more than 7 eggs per female per day over a 10-day period. This oviposition rate was almost as high as that obtained by allowing weevils to feed on fresh cotton squares. These results will be reported in more detail elsewhere.

Literature Cited

(1) Brazzel, J. R., Davich, T. B., Raven, K., Texas Expt. Sta., Misc. Pub. **353** (1959).
(2) Dustin, J. P., Czajkowska, C., Moore, S., Bigwood, E. J., *Anal. Chim. Acta* **9,** 256–62 (1953).
(3) Earle, N. W., Gaines, R. C., Roussel, J. S., *J. Econ. Entomol.* **52,** 710–12 (1959).
(4) Everett, T. R., Earle, N. W., *Ibid.*, in press.
(5) Fels, G., Veatch, R., *Anal. Chem.* **31,** 451–2 (1959).
(6) Kanchakk, A. A., *Biokhimiya* **26,** 393–8 (1961).
(7) Moore, S., Spackman, D. H., Stein, W. H., *Anal. Chem.* **30,** 1185–90 (1958).
(8) Moore, S., Stein, W. H., *J. Biol.*

Chem. **211,** 907–13 (1954).
(9) Plaisted, P. H., *Contrib. Boyce Thompson Inst.* **20,** 83–107 (1959).
(10) Vanderzant, E. S., *J. Econ. Entomol.* **56,** 357–62 (1963).
(11) Vanderzant, E. S., Davich, T. B., *Ibid.*, **54,** 923–8 (1961).
(12) Vanderzant, E. S., Richardson, C. D., Davich, T. B., *Ibid.*, **52,** 1138–43 (1959).

Received for review February 28, 1963. Accepted May 13, 1964. Mention of proprietary products in this paper does not constitute their endorsement by the U. S. Department of Agriculture.

INSECTICIDE ACTIVITY AND STRUCTURE

N-Alkyl Carbamates as Insecticides and Pyrethrins Synergists

H. I. THAYER, R. J. HARTLE, and A. MALLIS

New Products Division, Gulf Research & Development Co., Pittsburgh 30, Pa.

Of a series of *N*-alkyl carbamates three were found to show appreciable insecticidal activity against houseflies: 4-indanyl, 5-indanyl, and 3,4-methylenedioxyphenyl *N*-methyl carbamate. Other carbamates which were not toxic were effective synergists for pyrethrins and allethrin against cockroaches and houseflies; one outstanding example of this type was 3,4-methylenedioxyphenyl *N*-butoxyethoxyethyl carbamate.

For some time certain carbamates have been known to possess physiological activity, but particular interest in this class of compounds as pesticides has grown rapidly since 1947 (*19, 20*). Various aryl and heterocyclic esters of *N*-alkyl and *N,N*-dialkyl carbamates have been shown to be toxic to houseflies as well as aphids, thrips, mosquito larvae, and other insects (*7, 11, 12, 18, 21*, and others). Also, the pyrethrins synergistic activity of carbamates to houseflies has been investigated and reported in a few instances. Prill (*24, 25*) has claimed certain 3,4-methylenedioxyphenylalkyl *N*-alkyl carbamates to be pyrethrins synergists. In contrast, several 3,4-methylenedioxyphenyl *N*-aryl carbamates show only slight synergism for pyrethrins (*1, 2, 9*).

Our original interest was in determining the effectiveness of the indanyl moiety in a carbamate type of insecticide or pyrethrins synergist. Because the 5-indanyl and 3,4-methylenedioxyphenyl groups are similar to one another with respect to the spatial relations of their atoms, indanyl derivatives might show insecticidal or synergistic activity comparable to that of 3,4-methylenedioxyphenyl derivatives. Our second objective was to determine the effectiveness of Oxo-octyl—that is, mixture of octyl isomers obtained in the Oxo process (*17*)—derivatives of carbamates. Both the alcohol and the amine mixtures were used in the preparation of these compounds.

Experimental

Syntheses. The compounds evaluated in this study, listed in Tables I and II, were synthesized by one of two methods:

Method A. $R'NCO + ROH \rightarrow R'NHCOOR$

Method B. $R'NH_2 + ROCOCl \rightarrow R'NHCOOR$

or $R_2'NH$ or $R_2'NCOOR$

Choice of Method A or B was determined by the availability of starting materials. Methyl, ethyl, *n*-butyl, *n*-undecyl, and *n*-octadecyl isocyanates were obtained from commercial sources. *n*-Heptyl and *n*-butoxyethoxyethyl isocyanates were prepared from acid chlorides (*16*). With the exception of 3,4-methylenedioxybenzyl alcohol (*5*), all of the alcohols, amines, and phenols were purchased.

Method A. The synthesis of butoxyethoxyethyl isocyanate (IV) and the carbamate, S-16, formed by the reaction of IV with 3-4-methylenedioxyphenol (sesamol) illustrates this procedure. The reactions involved in the preparation of the isocyanate are as follows (*6*):

$$C_4H_9OCH_2CH_2OH + CH_2{=}CHCN \xrightarrow{\text{Base}} C_4H_9OCH_2CH_2OCH_2CH_2CN \quad (I)$$

$$\downarrow \text{HCl}, H_2O$$

$$C_4H_9OCH_2CH_2OCH_2CH_2COCl \xleftarrow{SOCl_2} C_4H_9OCH_2CH_2OCH_2CH_2CO_2H \quad (II)$$

(III)

$$\downarrow NaN_3$$

$$C_4H_9OCH_2CH_2OCH_2CH_2CON_3 \xrightarrow{\Delta} C_4H_9OCH_2CH_2OCH_2CH_2NCO \quad (IV)$$

3-(2-*n*-Butoxyethoxy)propionitrile (I). This compound was prepared by the base-catalyzed addition of butoxyethanol to acrylonitrile following the general procedure of Bruson (*3*).

To a mixture of 237 grams (2 moles) of butoxyethanol (dried over calcium hydride and distilled) and 3 ml. of a 35% solution of Triton B in methanol were added 106 grams (2 moles) of freshly distilled acrylonitrile. The addition was carried out at a rate to maintain a reaction temperature of 45° to 50° C. The mixture was stirred at room temperature for 1 hour after addition was complete and then made slightly acidic with acetic acid and filtered. The filtrate was distilled at reduced pressure to yield 293 grams (85%) of 3-(2-*n*-butoxyethoxy)-propionitrile, boiling at 95–97° C. at 1 to 2 mm.; $n_D^{25} = 1.4280$.

Analysis. Calculated for $C_9H_{17}O_2N$: C, 63.12; H, 10.00; N, 8.18. Found: C, 63.07; H, 10.17; N, 7.99.

3-(2-*n*-Butoxyethoxy)propionic Acid (II). 3-(2-*n*-Butoxyethoxy)propionitrile (257 grams, 1.5 moles) was added dropwise with stirring to 300 grams of concentrated hydrochloric acid which was held at 70° C. The mixture was stirred at this temperature for 3 hours, followed by 30 minutes of heating at 100° C. Water was then removed from the reaction mixture in a flash evaporator. The by-product, ammonium chloride, was separated by filtration and washed with ether. The solvent was distilled from the filtrate, and the product was distilled at reduced pressure to yield 237 grams (83%) of 3-(2-*n*-butoxyethoxy)propionic acid (boiling point, 134° C. at 1.5 mm.).