α-AMINOADIPIC ACID AS A CONSTITUENT OF A NATURAL PROTEIN

Thesis by

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SUMMARY

Thirty-five proteins or protein-containing materials were analyzed for α-aminoadipic acid by means of chromatography on starch columns. Of these materials corn and Taka-diastase (from Aspergillus oryzae) were shown to contain free α-aminoadipic acid.

As a protein constituent aminoadipic acid was found in a water-soluble corn protein. Corn steepwater concentrate was an adequate source of this protein. α-Aminoadipic acid was shown to be a constituent of the protein by its isolation from a hydrolysate. Its identity with the synthetic amino acid was proved by elementary analysis, mixed melting point, chromatographic behavior on both starch and Dowex-50 columns, use as a growth substance for a Neurospora crassa mutant which requires it for growth, and inhibition of growth by a specific inhibitor.

The ionization constants for aminoadipic acid in water were determined.

The conversion of radioactive α-aminoadipic acid into radioactive lysine in a mutant strain of Neurospora crassa was shown.

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INTRODUCTION

The earliest mention of α-aminoadipic acid as a metabolite in biological material was made in relation to transaminase systems by Braunstein (1). He learned that both pigeon breast muscle brei and a purified glutamic transaminase extracted from it were active in aminating ketoadipic acid to aminoadipic acid using alanine as a source of amino groups. No attempt was made to isolate the aminoadipic acid. An aspartic transaminase from a plant source was inactive.

Experiments by Borsook and Dubnoff (2) on the synthesis of arginine by rat kidney slices showed that in addition to the activity of glutamic and aspartic acids in transferring amino groups to citrulline lysine also was active. The authors suggested at that time that lysine was converted to glutamic acid. However, in light of the activity of aminoadipic acid in transamination Braunstein suggested that the lysine activity was due to its conversion into aminoadipic acid and this was the amino group donor (3). In view of this suggestion and the experimental results discussed in the next paragraph Dubnoff and Borsook (4) then repeated their previous experiments using aminoadipic acid as a substrate and found that it could indeed aminate citrulline (as measured by increase in arginine) sufficiently well to explain the activity of lysine.

By the use of radioactive compounds synthesized with C^{14} Borsook et al (5) presented conclusive evidence that one path of enzymatic lysine degradation in guinea pig liver homogenate was through α-aminoadipic acid, this compound being further degraded by oxidative deamination to α-ketoadipic acid, and then oxidatively decarboxylated to glutaric acid (6). Only L-lysine was found to be active. All the intermediaries were isolated, and characterized by elementary analyses, constant specific activities and melting points. This work was the first unequivocal demonstration of the natural occurrence of α-aminoadipic acid in biological material.

This raised the possibility of aminoadipic acid acting as a precursor for lysine in animals, and replacing lysine as an essential amino acid. To test this possibility Geiger and Dunn (7) fed a diet deficient in lysine to infantile rats. The rats grew normally when lysine was added, but failed to grow when an equivalent amount of aminoadipic acid replaced the lysine. The racemic and the optically active amino acid gave the same negative results. Leuconostoc mesenteroides and Streptococcus faecalis, bacteria which require lysine and are used in microbiological determinations of this amino acid, both failed to grow on aminoadipic acid. Similar results were independently found by Stevens and Ellman (8).

The only other work using aminoadipic acid as a metabolite is that reported by Mitchell and Houlahan (9) on a mutant strain of Neurospora crassa. Of the four lysine-requiring mutants known, one (strain 33933) utilizes aminoadipic acid in place of lysine, thus placing this amino acid in the role of a possible intermediate in the biosynthesis of lysine. If given in the presence of equivalent quantities of the L-form the D-isomer is utilized as well as the L-form. Furthermore, the cyclized form (2-carboxy-6-piperidone), although unable to initiate growth, is completely utilized in the presence of the L-form. In light of these facts it is difficult to understand the complete inactivity of α-ketoadipic acid in either initiating or supplementing growth started by L-α-aminoadipic acid.

The presence in cholera Vibrio of aminoadipic acid as well as hydroxy-aminoadipic acid had been reported by Blass and Macheboeuf (10) but this claim was later renounced by the authors (11) on the basis of contamination of the isolated product by leucine, valine, alanine, and possibly glycine, as shown by paper chromatography. Using another method the author (12) confirmed the absence of the new amino acid from the cholera organism. That Asiatic cholera could synthesis aminoadipic acid from ketoadipic acid and ammonia was claimed by Yacobson et al (13), this property being unique among the organisms tested. No attempt was made to isolate the final product. The increase in amino

nitrogen was measured by the Van Slyke nitrous acid method. In a later report (14) greater validity was given to the claim by the inclusion of paper chromatograms showing the presence of aminoadipic acid in the reaction mixture.

The occurrence of free aminoadipic acid in biological materials raises the possibility that this amino acid may be a constituent of larger nitrogen-containing compounds, including proteins. In the research described herein evidence is presented that α-aminoadipic acid is a constituent of a naturally occurring protein.

SURVEY METHODS

No unique chemical properties of aminoadipic acid are known which serve to differentiate it well enough from glutamic acid and aspartic acid to serve as the basis of a quantitative method for its determination.

The only micro-organism known which could be used in a bio-assay is the earlier-mentioned lysineless mutant of Neurospora crassa which utilizes aminoadipic acid for growth. However, growth of this organism is inhibited by the presence of glutamic acid or aspartic acid, both of which are present in comparatively large quantities in most protein hydrolysates. The organism is thus unsuitable for a bio-assay.

However, chromatography on starch columns by the method of Stein and Moore (15), eluting with a propanol-butanol-hydrochloric acid solution has been shown by

Borsook *et al* (5), and Moore and Stein (16) to separate glutamic acid and aminoadipic acid from one another sharply and reproducibly. The method is sufficiently sensitive to determine as small a quantity as 10 micrograms of an amino acid in 2 milligrams or more material when used with the quantitative ninhydrin method of Moore and Stein (17). This was the method of choice for this investigation.

EXPERIMENTAL

Potato starch columns were prepared according to the method of Stein and Moore (15). Table 1 shows the sizes of columns used, all having a length of 30 cm.

Table 1

Starch Columns

Size	Diameter	Starch weight	Capacity
	cm.	gm.	mg.
Small	1.2	25	2.5
Intermediate	2.3	85	25
Medium	4.1	335	100
Large	7.6	1000	300

Proteins shown in Table 2 were examined for the presence of aminoadipic acid.

Table 2

Proteins analyzed

Higher animals	Source of supply
Casein (vitamin-free)	Labco #9762
Gelatin	Knox Sparkling
Egg albumin	Merck, #31047
Bovine hemoglobin	H. A. Itano
Human serum albumin	Fraction V, Harvard Med. School
Human γ-globulin	Fraction II, " " "
Cytochrome C	Nutrit. Biochem. Corp.
Crystalline zinc insulin	Eli Lilly Co., 987267
Pepsin, U.S.P.	City Chem. Corp. 7626
Trypsin	
Pancreatin	Eli Lilly Co. 2071-305175
Erepsin	Nutrit. Biochem. Corp.
Lipase (steapsin)	" " "
Keratin	Merck, 43196

Higher Plants	Source of supply
Gliadin	Bios Lab.
Zein	Nutr. Bioch. Corp. 1054
α-Zein	Corn Prod. Refin. Co.
β-Zein	" " " "
Corn glutelin	" " " "
Corn pollen	H. J. Teas

Table 2 (continued)

Higher Plants	Source of supply
Whole corn seed	H. J. Teas
Corn Steepwater	Corn Prod. Refin. Co. 5203
Urease	Nutr. Bioch. Corp. 1607
Tobacco leaf cytoplasmic protein	S. Wildman
Almond meal	S. B. Penick Co.
Papain, N. F.	Merck 41678
Ricin, C. P.	Eimer Amend D29
Lower Organisms	
Cholera *Vibrio*	Eli Lilly Co. E-591
Escherichia coli	A. Siegal 7
Ulva	H. J. Teas
Nereocystis Luetkeana	" " "
Neurospora crassa	M. Fling
Lyphophora Williamsi	H. J. Teas
Tobacco mosaic virus	S. Wildman
Taka-diastase (*Aspergillus oryzae*)	Parke Davis Co.

A known weight of protein was hydrolyzed with a minimum of 40 parts of 20% HCl, refluxing on an oil bath for 20 hours. To an aliquot of hydrolysate representing 2.5 mg. of protein was added radioactive α-aminoadipic acid (9.3 μg., 130 c.p.m.) and excess HCl repeatedly evaporated

off *in vacuo*. The marked aliquot was transferred to the top of a small starch column in 0.5 ml. 1:2:1 solution (1 part n-butanol, 2 parts n-propanol, and 1 part 0.1 N HCl), driven into the starch under 15 cm. mercury pressure, and washed in with 0.2 ml. 1:2:1 solution. The column was placed on an automatic fraction-collector adjusted to deliver 0.5 ml. fractions and a reservoir of 1:2:1 solution attached to the column under 15 cm. mercury pressure.

Odd-numbered fractions were analyzed for amino nitrogen by the Stein and Moore quantitative ninhydrin method (17) and the even-numbered ones evaporated to dryness in aluminum cups and the radioactivity measured with a Geiger-Muller counter. It was soon noticed, as shown in Table 3, that a constant ratio existed between the volumes of eluate at the aminoadipic acid peak (radioactivity) and the proline peak (yellow color with ninhydrin).

Table 3

Effluent Ratio

Protein	Fraction Number at Peak		Ratio
	Aminoadipic acid	Proline	
Casein	89	113	0.79
Gliadin	104	130	0.80
Zein	84	106	0.79
Cholera Vibrio	54	69	0.78
	Average		0.79

Although the volumes of eluate varied with the salt content of the hydrolysates and the water content of the starch, the ratio between the peaks remained constant. It was thus possible to conserve the limited supply of radioactive amino acid and rely on the position of the proline peak to locate the aminoadipic acid peak.

The majority of proteins tested showed no aminoadipic acid peak greater than the amount of radioactive tracer added, with the exception of the proteins to be discussed below. Figure 1 illustrates the spectrum of a crystalline zinc insulin hydrolysate to which 50 μg. of proline had been added as a marker. The position of the aminoadipic acid locus was calculated from the proline peak. No trace of aminoadipic acid was found.

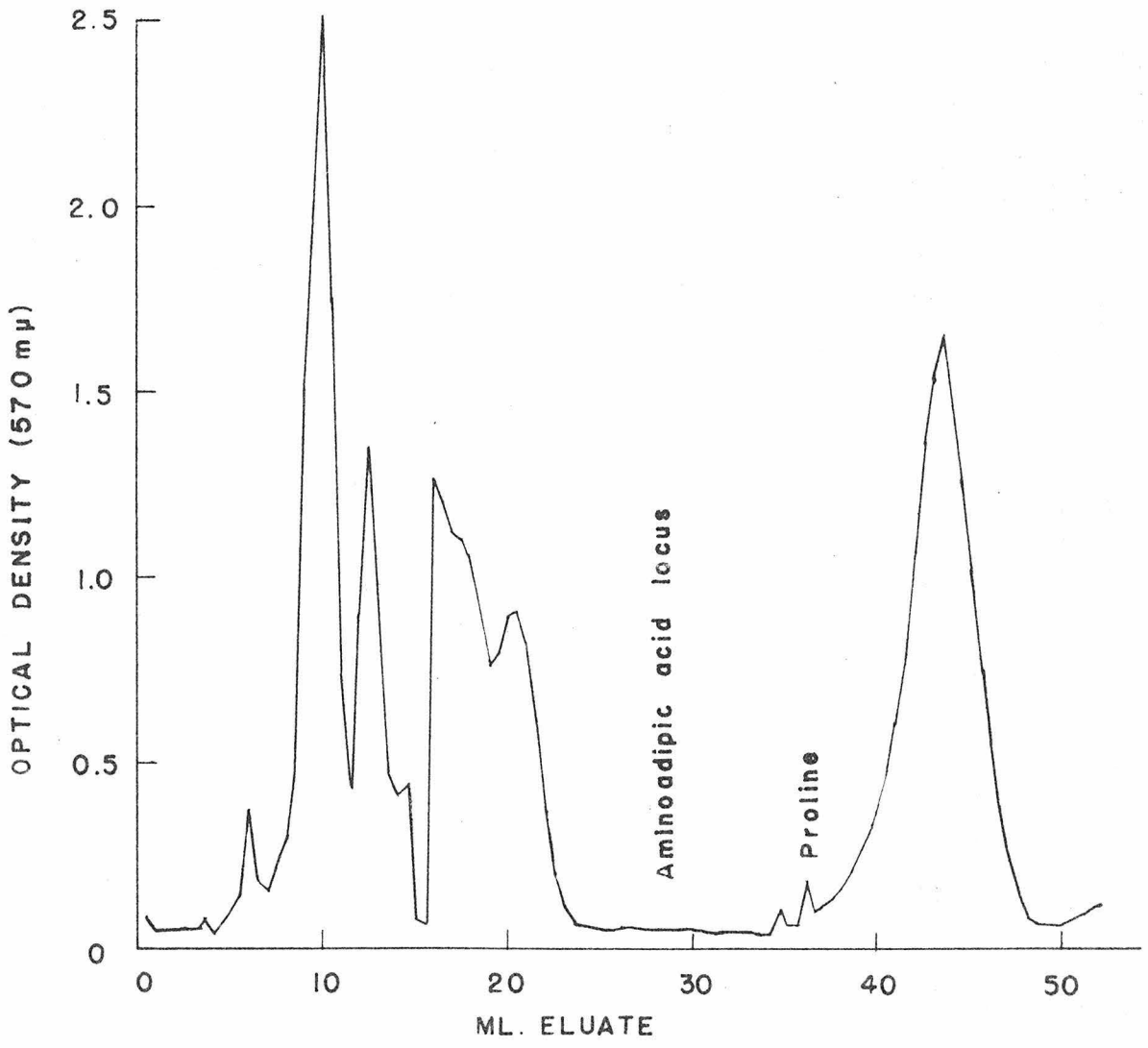
Protein hydrolysates which showed the presence of aminoadipic acid when run on a small starch column were analyzed on larger starch columns, the eluate in the aminoadipic region evaporated to dryness, the esters formed with propanol and butanol hydrolyzed with HCl, excess HCl removed, and the residue rechromatographed on a small starch column. If a ninhydrin peak again appeared in the proper location for aminoadipic acid it was considered a real effect.

FIGURE 1

CRYSTALLINE ZINC INSULIN HYDROLYSATE

(2.5 mg. + 50 µg. Proline)

on small starch column



Analysis of Zein

When the amino acids in Zein hydrolysates were thus separated on starch columns and analyzed by the quantitative ninhydrin method there was found 0.07% aminoadipic acid (Figure 2). More highly purified samples of zein were obtained, designated as α-zein, soluble over a wide range of alcohol-water concentrations, and β-zein, soluble in 55 to 75% alcohol, and differing in their sulfur content. Neither was found to contain aminoadipic acid. This suggested that the aminoadipic acid previously found in zein had been present as an impurity and that other protein fractions from corn might contain it in larger amounts. Accordingly a sample of 28-day whole corn seed was obtained and analyzed for aminoadipic acid. The whole corn seed was found to contain 0.15% (Figure 3).

Fractionation of Corn Seed

The corn seed was then separated into five fractions on the basis of solubilities, according to a modification of the fractionation scheme of Zeleny (18)(Table 4). Three grams of 28-day whole corn seed ground to 40 mesh in a Wiley mill were extracted 18 hours in a Soxhlet extraction apparatus with ethyl ether. The ether was removed from the corn and the dried product then put into a dialysis sac and dialyzed against several changes of distilled water until all ninhydrin-reacting material capable of diffusing through the cellophane had been removed.

FIGURE 2

ZEIN HYDROLYSATE

(250 mg.)

on large starch column

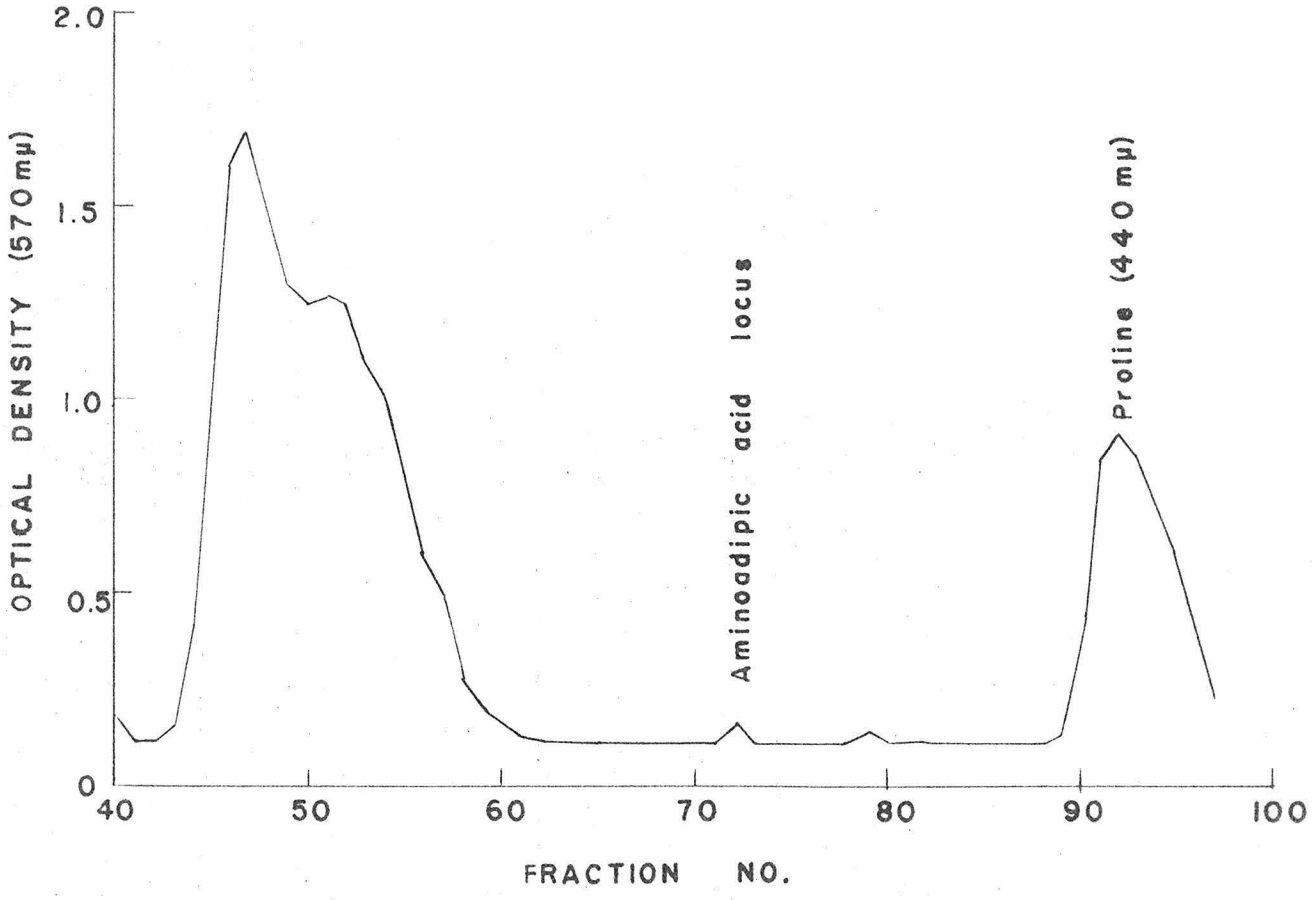
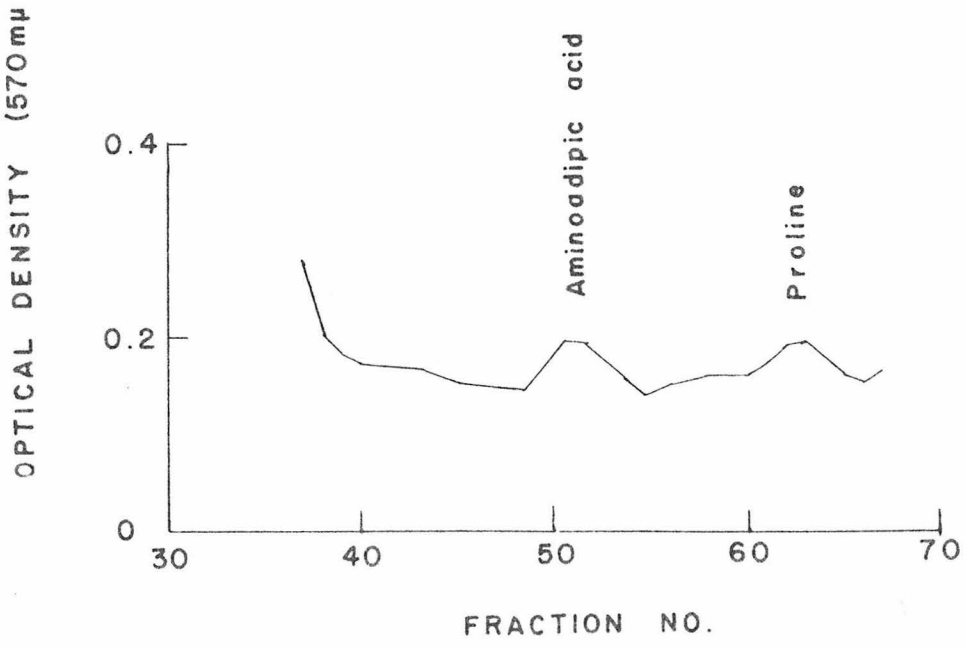


FIGURE 3

CORN HYDROLYSATE

(10 mg.)

on small starch column



The material in the dialysis sac was filtered, the filtrate and washings evaporated to dryness *in vacuo*, 30 ml. of 20% HCl added to it, and refluxed for 20 hours. This was Fraction 1, water-soluble non-dialyzable material.

Fraction 2-- The dried residue from the dialysis and filtration was suspended in 100 ml. N NaCl solution, shaken continuously for 3 hours at room temperature, and allowed to stand 21 hours longer; it was then filtered, and the insoluble portion on the filter paper washed twice with N NaCl. The filtrate and washings were combined, the volume reduced *in vacuo* and dialyzed against running water for 24 hours to remove the salt. Material in the dialysis sac was evaporated to dryness, and hydrolyzed with 20% HCl 20 hours.

Fraction 3-- The insoluble residue from the NaCl extraction was washed with water until all chloride had disappeared from the washings, and dried in air. It was then suspended in 100 ml. 80% ethanol for 24 hours, the first three hours with constant shaking, then filtered, and washed with 100 ml. 80% ethanol. The filtrate and washings were combined, ethanol evaporated off *in vacuo*, and the residue hydrolyzed with 20% HCl for 20 hours.

Fraction 4-- The insoluble residue from the alcohol extraction was dried, suspended in 100 ml. 0.2% NaOH for 24 hours, the first 3 with constant shaking, and filtered. The insoluble portion was washed with 100 ml. 0.2% NaOH, the filtrate and washings combined and evaporated to dryness *in vacuo*, and hydrolyzed with 20% HCl for 20 hours.

Fraction 5-- The insoluble residue from the NaOH extraction was dried, and hydrolyzed in 20% HCl for 20 hours.

Nitrogen determinations were made on the five fractions by micro-Kjeldahl and direct Nesslerization.

Table 4

Fractionation of Whole Corn Seed

Fraction	Soluble in	Nitrogen	Protein*	Part of Total protein	Part of Total corn seed
		mg.	mg.	%	%
1	Water	11.4	71.0	24.0	2.4
2	N NaCl	2.7	17.0	5.8	0.6
3	80% Ethanol	4.5	28.4	9.6	1.0
4	0.2% NaOH	10.4	65.3	22.1	2.2
5	Insoluble	18.2	113.7	38.5	3.8
TOTALS		47.2	295.4	100.0	10.0

*The factor 6.25 was used.

Each fraction was then analyzed for aminoadipic acid. The water-soluble non-dialyzable fraction contained 0.06%. (Figure 4). The other fractions were free of aminoadipic acid.

Fractionation of Steepwater Concentrate

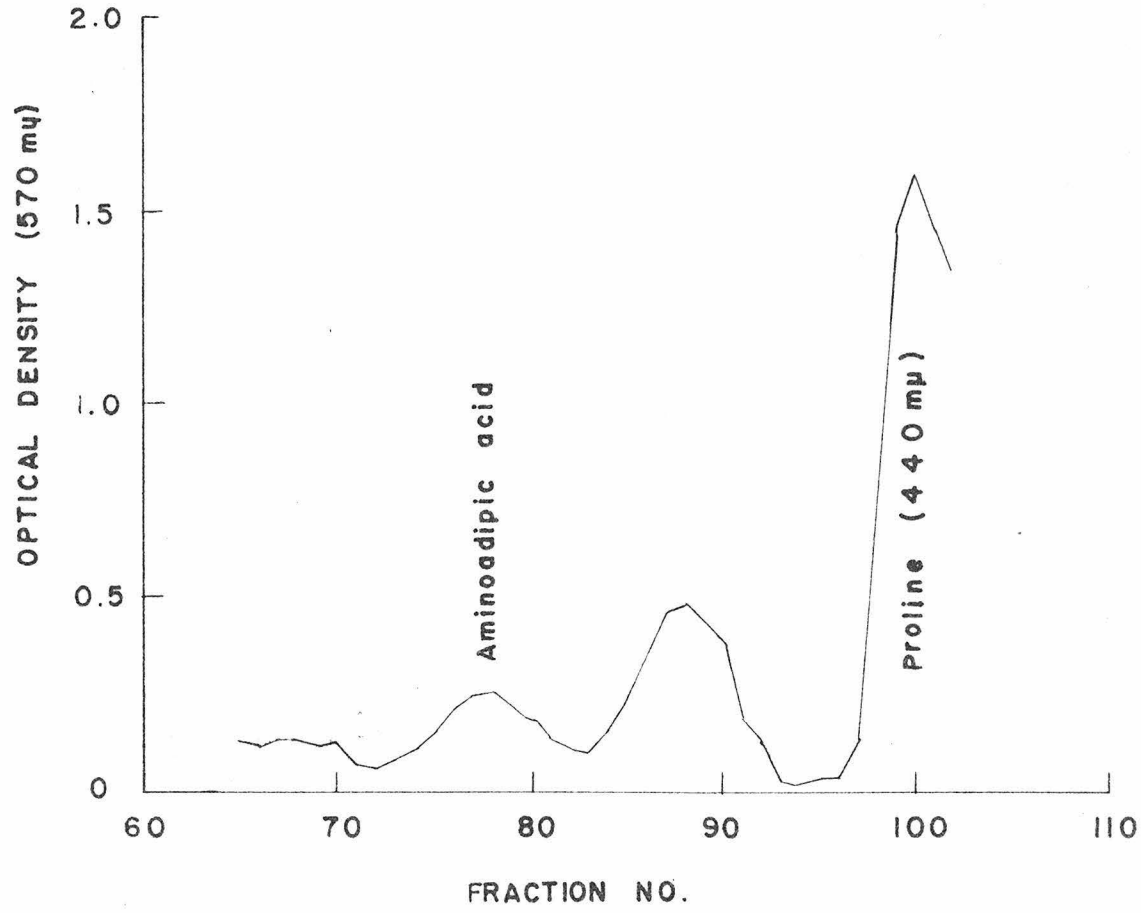
The next step was to find an abundant source of the water-soluble constituents of corn. Corn steepwater concentrate, a by-product of the corn-milling industry, was available and seemed to have possibilities as a source of the soluble portions of corn. Several kilograms were ob-

FIGURE 4

WATER-SOLUBLE CORN PROTEIN

(Concentrate from medium column)

on small starch column



tained and analyzed for moisture, ash, and nitrogen (Table 5).

Table 5

Composition of Steepwater Concentrate

Specific gravity	1.25
Dry matter	53.8%
Ash (dry basis)	18.7%
Total N (Dry basis)	8.3%
pH	3.9

A 100 mg. aliquot of the steepwater was hydrolyzed with 20 ml. of 20% HCl for 20 hours, evaporated to dryness, and analyzed on an intermediate starch column. The eluate in the aminoadipic acid region of the elution spectrum was refluxed with HCl to hydrolyze its esters formed with propanol and butanol, 18.6 μg. radioactive aminoadipic acid (260 c.p.m.) added, the mixture evaporated to dryness, taken up in 1:2:1 solution and put in a small starch column. The radioactive peak in the eluate contained 132 μg. aminoadipic acid. Thus free plus combined aminoadipic acid totalled 0.13% of the steepwater (Figure 5).

Similarly, an unhydrolyzed 100 mg. portion was analyzed and found to contain 72 μg. or 0.07% aminoadipic acid.

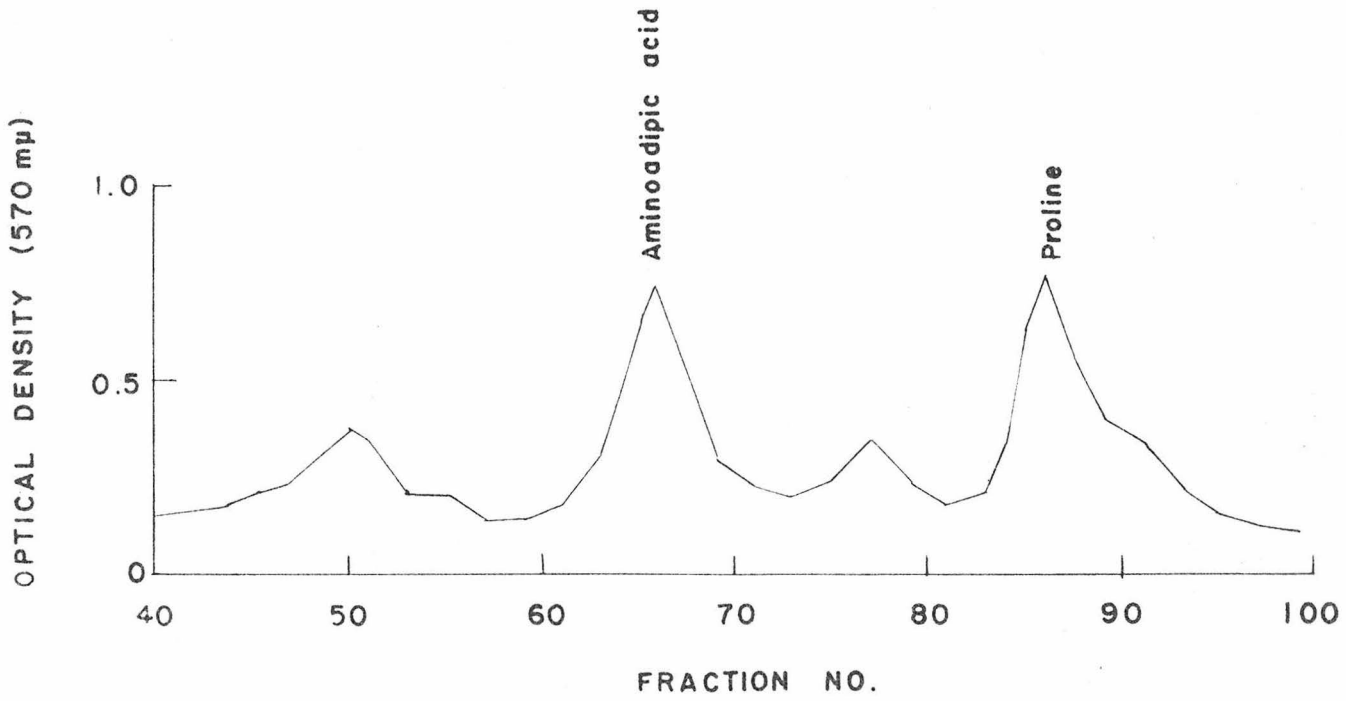
The steepwater was fractionated to determine the distribution of aminoadipic acid (Table 6).

FIGURE 5

STEEPWATER HYDROLYSATE

Intermediate column eluate

on small starch column



Protein fraction-- 70 g. steepwater was precipitated with trichloroacetic acid having a final concentration of 10%; the precipitate was washed with water, acetone, and ether, the latter removed by heat. The protein was hydrolyzed, and analyzed for aminoadipic acid (Figure 6).

Non-protein non-dialyzable fraction-- The filtrate and washings from the protein precipitation were combined, reduced in volume, placed in a dialysis sac and dialyzed at 5° C. against 4 changes of distilled water containing 0.1% phenol to inhibit bacterial action. The contents of the sac were then hydrolyzed, after being reduced in volume.

Dialysate-- The dialysate was concentrated to a small volume, and an aliquot representing 200 mg. steepwater analyzed for aminoadipic acid (Figure 7).

Table 6

Aminoadipic acid content of Steepwater Fractions

Fraction	Aminoadipic Acid
Total (hydrolyzed)	0.13%
Free (unhydrolyzed)	0.08
Protein (hydrolyzed)	0.05
Non-dialyzable, non-protein (hydrolyzed)	$<$0.01

FIGURE 6

STEEPWATER PROTEIN HYDROLYSATE

on intermediate starch column

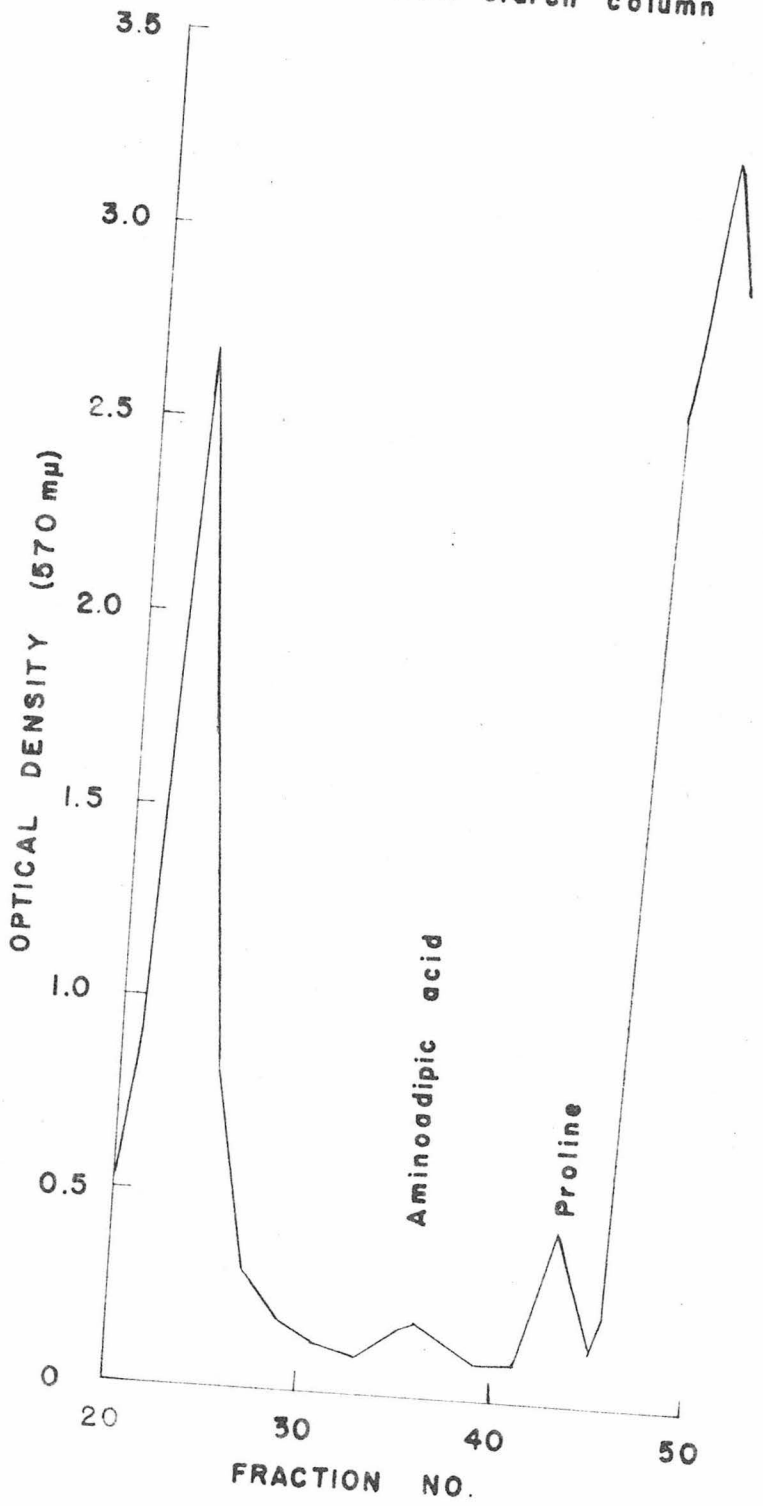
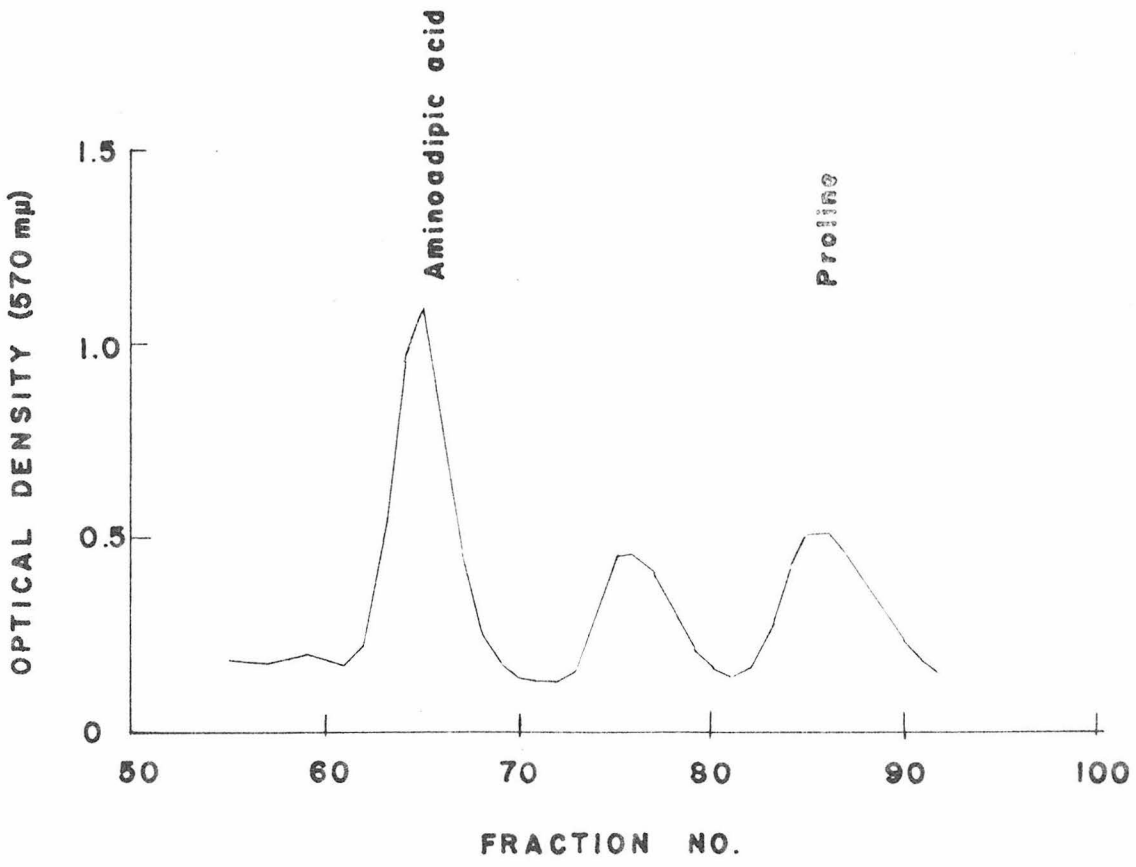


FIGURE 7

STEEPWATER DIALYSATE

Intermediate column eluate

on small starch column



Isolation of aminoadipic acid from steepwater protein

A 2 Kg. quantity of steepwater was diluted to four times its volume and strong NaOH solution added with stirring until the pH was raised to 5.0. The protein was precipitated by boiling for 30 minutes, constantly stirring to avoid bumping, and was then allowed to cool to room temperature. The coagulated protein was filtered on a large Buchner funnel, and washed with copious quantities of water, 95% alcohol, and ether. Ether was removed in a drying oven at 105° and the protein dried to constant weight. Protein tests with Millon's reagent and the Hopkins-Cole reagent were positive, as were the xanthoproteic reaction and the biuret test. Yield: 253 g., 12.7%.

The protein was ground to a powder in a mortar, placed in a 3 liter flask, and hydrolyzed with 1 liter of 20% HCl for 20 hours on an oil bath. Excess HCl was removed *in vacuo*, water repeatedly added and evaporated off to remove as much HCl as possible. In order to precipitate the dicarboxylic amino acids according to the method of Chibnall *et al* (19) a slurry of calcium hydroxide in water was added until the reaction was alkaline, the mixture filtered to remove undissolved calcium hydroxide and the volume of the filtrate and washings reduced to 400 ml. The solution was added to 3400 ml. of absolute alcohol, a copious precipitate forming immediately. This was filtered, washed with absolute alcohol, and dissolved in water. The precipitation

with calcium and absolute ethanol was repeated. The precipitate was dried and weighed. Yield: 14.7 g., 0.74% of the steepwater.

The calcium salts were dissolved in water, saturated oxalic acid solution was added until no more calcium oxalate would precipitate, and the precipitate discarded. Filtrate and washings were reduced to 120 ml. in vacuo.

A Dowex-50 column (a nuclear sulfonic acid polymer) was prepared according to the method of Stein and Moore (20) by pouring a slurry of 400 gm. of Dowex-50 in 0.5 N HCl into a glass tube (2.3 X 90 cm.) having a coarse sintered-glass plate at the bottom, and allowing 0.5 N HCl to drip through over-night. A 15 ml. aliquot of the dicarboxylic amino acids, to which 93 μg. radioactive aminoadipic acid (1300 c.p.m.) had been added, was placed on top of the Dowex-50 column in 0.5 N HCl. When all the solution had gone into the column it was washed in once with 0.5 N HCl, then a reservoir of 1.5 N HCl was allowed to drip into the column. The column was placed on an automatic fraction-collector adjusted to deliver 10 ml. fractions. After 24 hours the reservoir was changed to 2 N HCl and the collecting continued for 48 hours more. Aliquots of 0.5 ml. were taken from every alternate fraction and analyzed by the quantitative ninhydrin method after neutralizing with 5 N NaOH to methyl red.

In order to identify the amino acids, further 0.1 ml. aliquots of the fractions containing the ninhydrin-reacting peaks were placed on paper and chromatographed by the ascending method of Williams and Kirby (21), in phenol saturated with water. Each peak was tested for radioactivity. Thus it was learned that the aminoadipic acid was eluted from the Dowex-50 immediately after glycine and before alanine, slightly overlapping both of these amino acids (Figure 8). All the fractions containing radioactive material were pooled.

In an identical way the entire 120 ml. obtained from the Chibnall calcium hydroxide-ethanol separation were passed through a total of eight Dowex-50 columns. The pooled radioactive solution was evaporated to dryness and placed on a large starch column in 30 ml. of 1:2:1 solution, 15 ml. fractions being collected. The fractions containing the radioactivity were evaporated to dryness, esters hydrolyzed for 20 hours with 20 ml. of 20% HCl, excess HCl evaporated off in vacuo. In order to remove starch degradation products the residue was again taken up in 0.5 N HCl and placed on a Dowex-50 column, as described above. A single sharp peak was observed where the ninhydrin values and the radioactivity coincided. The ninhydrin values added up to 17.5 mg. of aminoadipic acid. There was no overlapping with other ninhydrin peaks, and the other peaks contained no radioactivity (Figure 9).

FIGURE 8

STEEPWATER PROTEIN CONC.

on Dowex-50 column

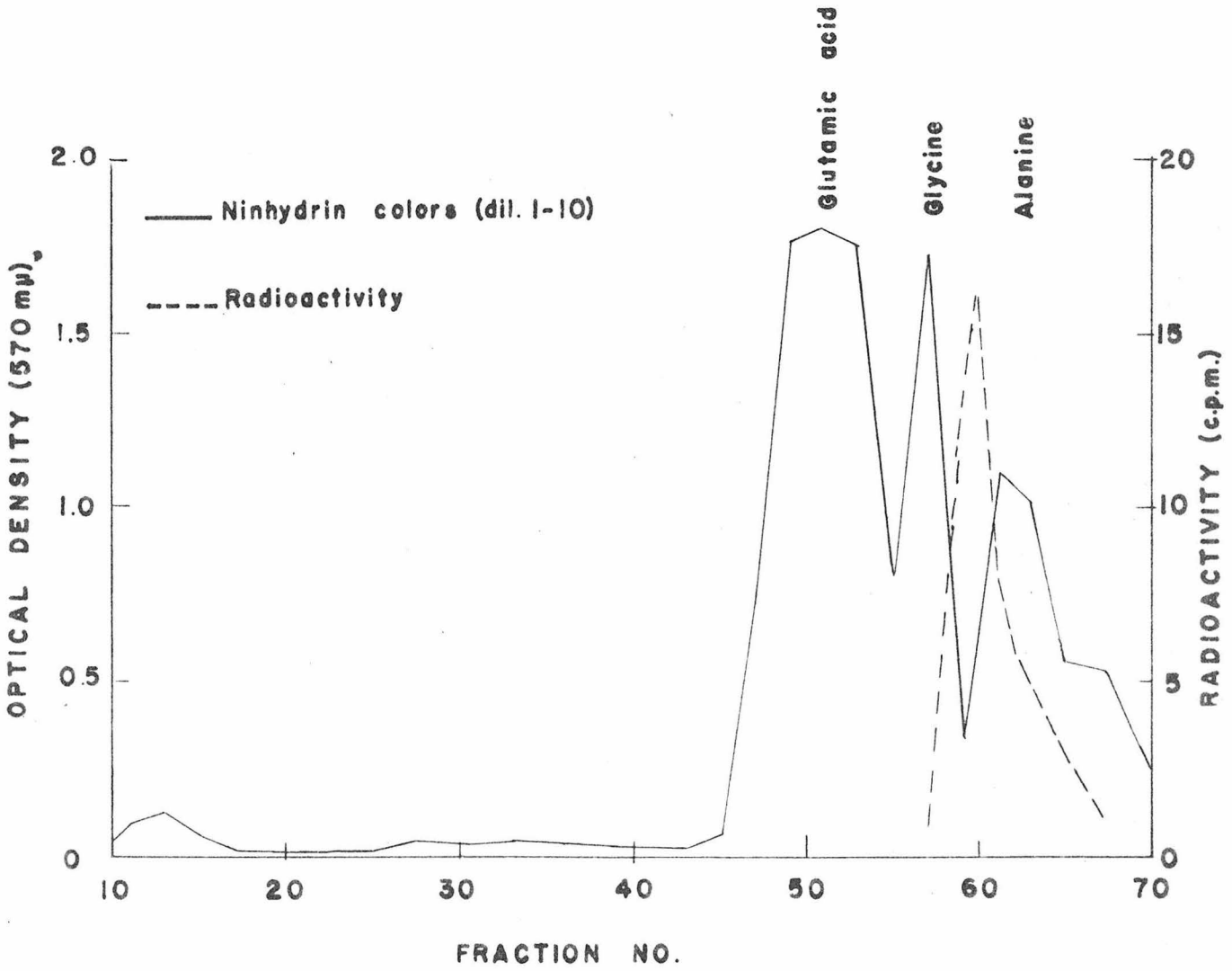
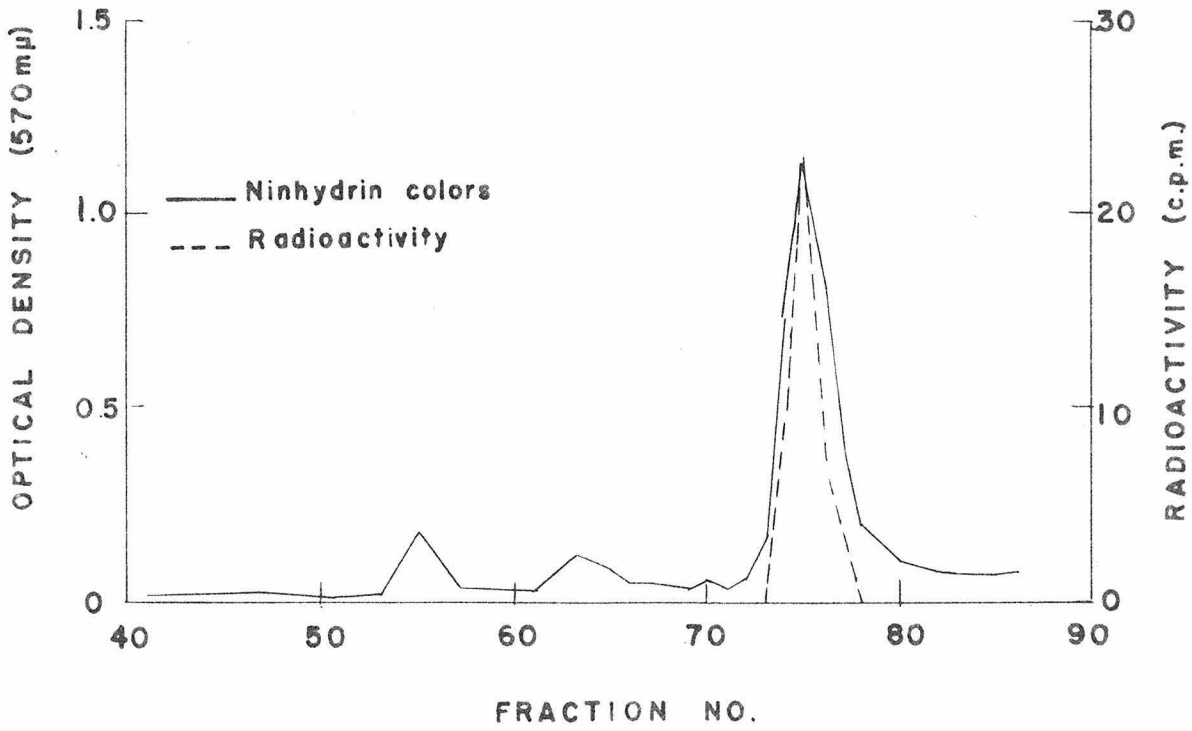


FIGURE 9

STARCH COLUMN CONCENTRATE

on Dowex-50 column



Crystallization

All the fractions in the peak were pooled and evaporated to dryness, water added, and again evaporated to remove all excess HCl. The residue was transferred to a beaker with 2 ml. of water, placed in a desiccator over solid KOH and the pressure lowered to 30 mm. by means of a filter pump. The yellowish solution was allowed to stand until macroscopic crystals appeared. These were too hygroscopic to weigh, so it was necessary to isolate the free acid.

The crystals were dissolved in 2 ml. of water, and the solution was titrated to pH 3.1 with 3 N ammonium hydroxide, 0.07 ml. being required. The electrodes and stirrer were washed with a small amount of water and the washings added to the solution. To remove the yellow color 5 mg. of acid-washed Norite was added to the solution and allowed to stand with occasional stirring for one hour. The Norite was filtered off, washed with a small amount of water, dried and counted for radioactivity. It had retained only 54 c.p.m.

The aminoadipic acid solution was evaporated to 0.5 ml. in a desiccator over concentrated sulfuric acid and fused potassium hydroxide, 3 ml. of 95% alcohol were added, and the crystals which appeared were allowed to grow overnight in the refrigerator. The next day the crystals were centrifuged off, washed with small portions of 95% alcohol and ether, and dried in air. Weight: 11.1 mg., specific activity: 213.6 c.p.m. per mg.

Five mg. of the crystals were redissolved in a small amount of water, 5 mg. Norite added and the solution was heated with occasional stirring for one hour. The solution was filtered, filtrate and washings reduced in volume to the saturation point and 3 ml. of alcohol added. The alcoholic solution was heated to boiling, and allowed to cool in the refrigerator overnight. The crop of crystals were filtered off, washed with small portions of alcohol, and ether, and dried. Weight: 3.0 mg., specific activity: 245 c.p.m. per mg. Melting point on an aluminum block: 206° with decomposition, mixed melting point with an authentic sample, 204-205° with decomposition.

Analysis - $C_6H_{11}O_4N$ (161.17) Calculated C 44.72, H 6.88, N 8.70; Found C 44.87, H 6.97, N 8.81

Titration curve

Aminoadipic acid (0.05 milliequivalent) was dissolved in 1.3 ml. water and titrated with 0.106 N HCl to pH 1.5 using a Beckman pH-meter, then titrated with 0.1112 N NaOH to pH 11.6 (Figure 10). The ionization constants were: pK_1 2.14, pK_2 4.21, pK_3 9.77, and pI 3.18.*

Neurospora growth

A Neurospora crassa mutant (strain 33933) grows on L- α-aminoadipic acid as well as lysine. However, growth on aminoadipic acid is inhibited by the presence of asparagine, while growth on lysine is stimulated by this amide (9).

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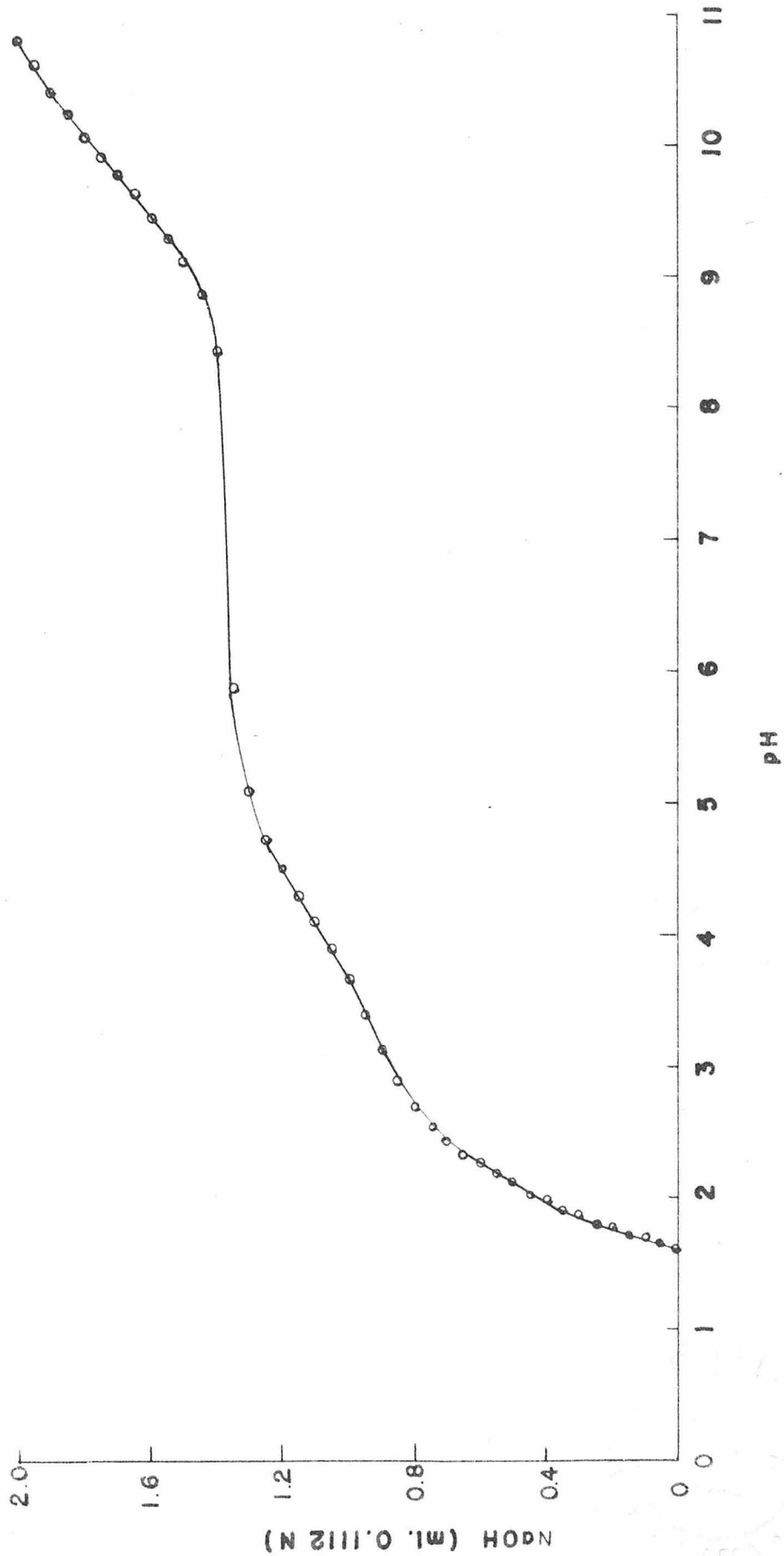
* See addendum, p. 33 b.

FIGURE 10

TITRATION CURVE, AMINOADIPIC ACID
(0.05 mM. in 2 ml. water)



Flasks containing 20 ml. of minimal medium plus varying amounts of the isolated aminoadipic acid were inoculated with a suspension of conidia of mutant 33933. To one flask was added asparagine. The mold was allowed to grow for four days at 25°. At the end of this time the mycelia were dried and weighed (Table 7), (Figure 11).

Table 7

Growth of mutant 33933 on aminoadipic acid

Aminoadipic acid		Mycelium
mMol	mg.	mg.
0	0	0
0.0025	0.40	0.6
0.005	0.80	4.2
0.01	1.61	13.9
0.015	2.41	27.8
0.02	3.22	41.0
0.005 plus 0.02 mMol asparagine		0

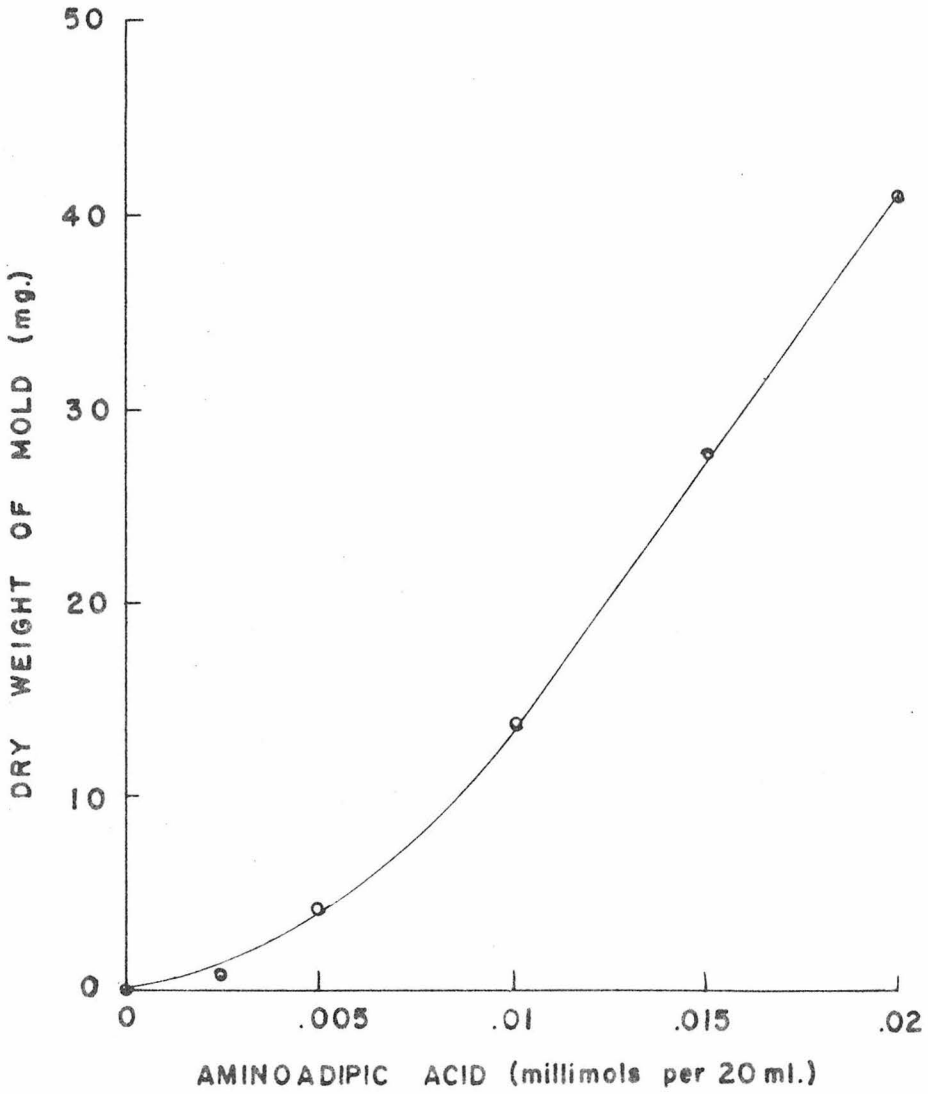
Radioactivity of lysine in Neurospora

The pooled mycelia (87.5 mg.) were ground to a powder, hydrolyzed with 40 ml. 20% HCl for 20 hours and the excess HCl repeatedly evaporated off *in vacuo*. The syrupy liquid was diluted to 0.6 ml., and an aliquot of 0.2 ml. put on the bottom of a sheet of Whatman No. 1 filter paper. Spots of aminoadipic acid and lysine were also put on the paper and the chromatogram developed for 16 hours with phenol

FIGURE 11

NEUROSPORA GROWTH

on aminoadipic acid



saturated with water. The paper was then dried at 100° and the part containing the known lysine, aminoadipic acid and the edge of the developed hydrolysate was sprayed with 0.1% ninhydrin solution in butanol saturated with water, and dried at 100° for 5 minutes.

The paper containing lysine from the hydrolysate was cut out, the lysine eluted with several small portions of water and the radioactivity of the eluate determined. The specific activity of the eluted lysine was 44.7 c.p.m. per μM. The aminoadipic acid used for growth had a specific activity of 42.7 c.p.m. per μM.

Analysis of Taka-diastase

A 10 mg. sample of Taka-diastase, a commercial water-extract of Aspergillus oryzae, was hydrolyzed with HCl. Radioactive aminoadipic acid (9.3 μg., 130 c.p.m.) was added as a marker and the hydrolysate was put on a starch column. The ninhydrin-reacting peak in the eluate which coincided with the radioactive peak contained 37 μg. of amino acid more than the added aminoadipic acid. This is 0.37% of the Taka-diastase. (Figure 12).

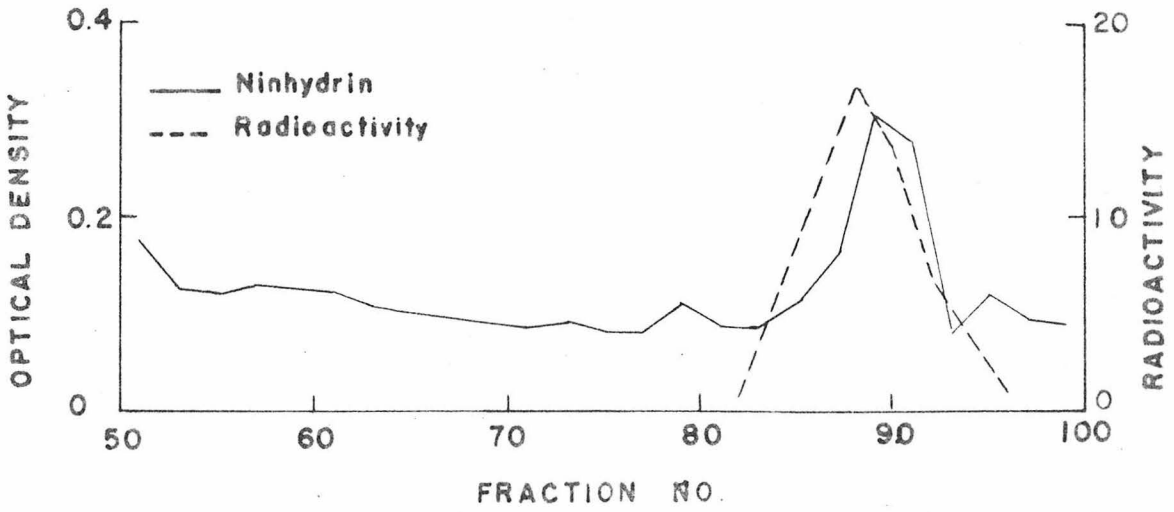
A larger sample (50 mg.) was dialyzed for 48 hours against running water. Material in the dialysis sac was reduced in volume, hydrolyzed with HCl and put on an intermediate starch column. No trace of aminoadipic acid was observed in the non-dialyzable portion.

FIGURE 12

TAKA-DIASTASE HYDROLYSATE

10 mg. plus 9.3 µg. aminoadipic acid

on small starch column



Titration curve, addendum

The following methods were used to determine the pK values:

(a). Midpoint between inflection points on Figure 10 (p.29)-- pK_1, pK_2, and pK_3.

(b). ΔpH was plotted against constant increase of standard alkali, and the pK was taken as the smallest value of ΔpH-- pK_2, and pk_3.

(c). pK_1 was calculated from an equation given by Schmidt (22) (Table 6b):

$$pK_1 = pH - \log\left(\frac{C}{A-(H^+)} - 1\right)$$

where A is concentration of acid, and

C is concentration of aminoadipic acid.

Table 6b

pK_1 of Aminoadipic acid

0.05 millimol aminoadipic acid in 1.3 ml. water, titrated with 0.106 N HCl, pH values from Beckman pH-meter with glass and calomel electrodes.

ml.HCl	pH	pK_1	ml.HCl	pH	pK_1	ml.HCl	pH	pK_1
0.12	2.70	2.09	0.32	2.20	2.15	0.54	1.89	2.20
0.14	2.62	2.09	0.34	2.18	2.19	0.58	1.83	2.15
00.15	2.60	2.11	0.36	2.15	2.20	0.62	1.77	2.06
0.16	2.58	2.13	0.38	2.11	2.18	0.70	1.70	2.14
0.18	2.52	2.14	0.44	2.03	2.22	0.74	1.68	2.09
0.20	2.44	2.10	0.48	1.97	2.23	0.78	1.65	2.07
0.24	2.36	2.14	0.50	1.92	2.15	0.90	1.58	2.07
0.30	2.23	2.14						
						AVERAGE		2.14

Ionization constants of glutamic acid (22): pK_1 2.19, pK_2 4.28, pK_3 9.66, pI 3.2

Aspartic acid: 2.09, 3.87, 9.82, 3.0

DISCUSSION

The requirements for the acceptance of amino acids published by Vickery and Schmidt (23) have been met in large part for α-aminoadipic acid. Identity between the isolated product and the synthetic amino acid was shown by elementary analysis, mixed melting point, identical chromatographic behavior on starch, Dowex-50, and paper, and growth of a Neurospora crassa lysine-less mutant which can grow on amino-adipic acid. The optical properties of the isolated amino acid were not investigated because the amount of material was too small. For the same reason derivatives were not prepared.

An unidentified ninhydrin-reacting substance was seen in the starch chromatograms of the water-soluble corn protein (Figs. 4 and 5) and the dialyzable portion of steep-water (Fig. 7), with its peak located between those of amino-adipic acid and proline.

The presence of aminoadipic acid in the water-soluble corn-seed protein cannot be taken as a unique property of corn until the seed proteins of other plant species are examined by the same techniques and found to be lacking.

The functions of aminoadipic acid in the protein may be two-fold: to join large peptides together, and to act as a precursor to lysine. Evidence for the first function is given by the very minute amount of aminoadipic acid

found in the non-dialyzable non-protein fraction of steep-water as compared with the amounts present as a free amino acid and bound in the protein form. Evidence for the second function is more indirect, and only be finally elucidated by direct experimentation. In Neurospora direct evidence was shown that aminoadipic acid was converted into lysine without dilution. It is possible that lysine formation proceeds in the same way in corn-seed protein. Stetten (24) has recently shown by the use of the isotopic amino acid that hydroxyproline of animal proteins is derived from the oxidation of proline already bound in the proteins and not from dietary hydroxyproline. A mechanism involving reduction and amination of aminoadipic acid to lysine is within the realm of possibilities.

If this is true the quantities of aminoadipic acid and lysine could vary, in the water-soluble protein, as a function of the age of the plant. One could expect that at an early stage of development more aminoadipic acid would be present in the protein than at a later stage.

Further work along the lines indicated would be desirable.

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