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FD-21

FLAVOR PRECURSORS IN MEAT

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W. A. LANDMANN, O. F. BATZER, A. T. SANTORO

SEPTEMBER 1965

AMERICAN MEAT INSTITUTE FOUNDATION
Chicago, Illinois 60637

Contract No. DA19-129-AMC-2114(X)

U. S. ARMY NATICK LABORATORIES
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FOREWORD

Processed meat items used in military feeding systems are often less acceptable than expected, in part because of deficiencies in desirable flavor. In order to develop process improvements to ensure full flavor in meat items it is necessary to have some knowledge about the mechanism of flavor generation. As a first step in attaining this knowledge, the identity of flavor precursors must be established.

The work covered in this report was performed by the American Meat Institute Foundation under Contract DA 19-129-AMC-2114(X) during the period from October 1962 to October 1963. It represents an attempt to establish the identity of substances extracted from beef muscle tissue which produce the desirable flavors and odors of cooked beef and to elucidate the mechanism by which the flavor is produced.

W. A. Landmann was the official investigator and O. F. Batzer and A. T. Santoro the collaborators in the research work for the American Meat Institute Foundation. The U. S. Army Natick Laboratories Project Officer was A. S. Henick of the Food Division.

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FLAVOR PRECURSORS IN MEAT

ABSTRACT

This report describes investigations of the precursor substances in beef muscle tissue which produce the desirable odors and flavors of cooked beef, in order to identify them and to elucidate the mechanism by which flavor is produced.

In earlier work, results showed that the dialyzable portion of a water extract of ground beef contained material, which, when heated in fat, produced a "broiled steak" odor, and when boiled in water, produced a beef broth flavor and odor. The type of material involved in this fraction included polypeptides, glucose, phosphates and inosinic acid.

The amino acid composition of the flavor fraction has been determined. Attempts to separate the phosphates for identification were unsuccessful because the isolated phosphate material could not be adsorbed on a Dowex-1 Cl^- column, even when the pH of the adsorbing solution was adjusted to 9.5.

Work with paper chromatography indicated that the phosphates were not glucose-1-phosphate or glucose-6-phosphate.

Improved separation of the flavor constituents on SE-sephadex C-25 resulted in a more concentrated fraction of the odor producing material. This fraction produced a strong odor, but a subsequent amino acid analysis indicated that its amino acid content was greatly decreased, in relation to previous fractions. Work must be continued to determine the chemical nature of the fraction obtained from SE-Sephadex C-25 resin.

Report

The purpose of this investigation was to identify and to determine the precursor substances in beef which give ise to the desirable odor and flavor of cooked meat. The work reported herein was a continuation of research which had been done prior to the initiation of the contract covering this research.

Observations had been made previously that the dialyzable fraction of a water extract of ground round of beef contained substances which produced a "broiled steak" odor when heated in fat, and a beef broth odor and flavor when boiled in water. Procedures for obtaining this fraction have been published (1).

A modification of the published procedure was used for the experiments described here, and is as follows: 1000 g. of round of beef, freed from all extraneous fat, was ground and then mixed with 2 liters of cold water. The slurry was allowed to stand in the cold (~5° C) for 3 hrs. with occasional stirring. The extract was filtered through a double layer of cheesecloth and the residue squeezed by hand. The residue was then mixed with 1 liter of cold water and the same process repeated. The two extracts were pooled.

Ten strips of dialysis tubing (Visking, 23 mm. in diameter 36 in. long) were washed in running tap water for 1 hour, then in distilled water (static, with several changes) for 30 minutes. The dialysis tubing was then filled with distilled water (~150 ml. in each tube) and suspended in the extract which had been placed in 2 liter graduate cylinders, and allowed to stand overnight (16 hrs. at 5°C.). The dialysis tubes were then removed from the extract, the exterior rinsed with running water very briefly, and the contents of the tubes placed in 500 ml. Erlenmeyer flasks (~200 ml. in each). The solutions were shell-frozen by rotating the flasks in a dry-ice-acetone bath and then freeze-dried in a Stokes freeze-drying apparatus. A white crystalline powder remained in the flasks. These were corked immediately and stored at -18° C. until used. Material was stable for from 6-8 weeks. The identification of some of the components contained in this fraction was carried out and results were reported in a second publication (2). As a result of these studies, three compounds were considered necessary for the production of the "broiled steak" odor: a glycoprotein-like material consisting of amino acids in peptide chains associated with glucose; phosphorus, as phosphate; and inosinic acid.

The material was analyzed for its amino acid content by hydrolysis with constant boiling hydrochloric acid overnight in a sealed tube at 100° C., and examination of this hydrolyzate in an automatic amino acid analyzer. Amino acids identified were histidine, 1-methyl histidine,

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proline, leucine, isoleucine, alanine, valine, serine, and β-alanine. Since the combined mole ratios of histidine and 1-methyl histidine were practically the same as that of β-alanine, it was assumed that these substances were originally present as carnosine and anserine. Subsequent paper chromatography on unhydrolyzed samples, and comparison to carnosine and anserine standards verified this assumption. Additions of these compounds to the original fraction did not enhance the odor produced and elimination of these compounds from the fraction did not decrease the apparent "broiled steak" odor.

In the procedure for obtaining the flavor fraction, as outlined in (1), a second dialysis step was used which eliminated a large amount of extraneous material. Due to the inability to obtain sausage casing that would duplicate the initial results, efforts were made to standardize this phase by modification of the cellulose casings according to the procedure of Craig and Konigsberg (3). After an apparent decrease in pore size on dialysis casing by the above procedure, when used, considerably more material remained in the casing after the second dialysis, but the "broiled steak" odor was not present when the material was heated in fat. Further efforts to modify the casings were abandoned when it was found that the flavor fraction with only a small amount of extraneous material could be obtained by the use of Sephadex G-25.

The fraction referred to in the above paragraph was obtained from ~200 mg. of material obtained by freeze drying the first diffusate. This amount was dissolved in 10 ml. distilled water, applied to a 2.5 X 30 cm column of Sephadex G-25, and eluted with distilled water. The column was prepared as described by Porath and Flodin (4). Fractions of 3 ml. were collected and the optical density of each at 290 mμ was taken. Readings were plotted on graph paper. A typical plot is presented in Figure 1. The tubes representing the fractions enclosed by the arrows on the diagram represent the location of material that produced the "broiled steak" odor when heated with fat. This fraction was very unstable and deteriorated rapidly at room temperature into a brown mass, even though it had been freeze-dried. This deterioration could be slowed down by storing the dried powder at 0° F. (-18° C.), at which temperature the material was stable for several weeks. Since this fraction contained an appreciable amount of phosphorus and since the findings of Wood (5) implicated sugar phosphates as the initiating agents in browning, attempts were made to determine the identity of the phosphates present in the "steak odor" fraction. The material obtained from the Sephadex run (~60 mg.) was dissolved in 100 ml. distilled water and brought to pH 8.0 with 0.1N sodium hydroxide. This solution was adsorbed on a 2.5 X 40 cm. column of Dowex 1 resin, Cl^- form, 8X, 200-400 mesh. After the adsorbing solution passed through, the column was washed with 100 ml. distilled water. A continuous gradient elution was then applied, starting with the pH of distilled water (~5.5) and ranging to that of 0.006N hydrochloric acid (~2.28). Practically all of the phosphorus, as well as the rest of the material, was not absorbed and was recovered in the adsorbing eluent and water wash. The combined

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adsorbing eluent and wash solution was lyophilized. The material was quite stable, indicating that the cause of the instability had been altered in some manner, or had been retained on the column. However, there was also an apparent decrease in the intensity of the "broiled steak" odor which was obtained when the eluted material was heated. The optical density of each fraction (10 ml) obtained by gradient pH elution was read at 290 mμ, and the solution in each tube was spot tested with ninhydrin for amino acid compounds, ammonium molybdate for phosphorus and p-aminohippuric acid for carbohydrates. Only one small peak was obtained by O.D. readings. This material was negative to ninhydrin and to ammonium molybdate, but was positive with p-aminohippuric acid. The amount of material obtained from the pooled fractions, represented by the curve, was too small to weight. No other material could be detected in any solutions emerging from the column, including a 2 N hydrochloric acid rinse at the end of the run.

The inability of the phosphorus containing material to exchange on Dowex-1 was considered rather unusual, and was indicative that the phosphate group is not free. The column experiment was tried several times, using increasingly higher pH's of the adsorbing solution. No appreciable adsorption occurred even when the pH of the solution was 9.5. It was felt that the pH could not be raised above 9.5 because deamination began to take place.

Attempts were made to identify the small amount of material that was obtained from the Dowex-1 column by gradient pH elution. Since the material eluted from the column at about pH 4.4 was negative for phosphate, the assumption that the material might be a carboxylic acid seemed valid. Micro tests on the freeze dried material showed the presence of nitrogen. The material was also positive for sugar. An ultra-violet spectrum was taken on a water solution of the material. A broad maximum at 275 mμ and a minimum around 258 mμ were recorded. A literature search revealed that a similar spectrum was produced by a substance obtained from heated neuraminic acid (6), later identified as 2-carboxypyrrole. Further examination showed that the U.V. spectrum of the unknown matched very closely the spectrum published by Gottschalk (7) for 2-carboxypyrrole. The unknown material also gave a positive test with Erhlirch's reagent, for indoles and arylamines. Up to this point, the results obtained strongly suggested that the unknown substance could be neuraminic acid or some substance similar to it.

Bial's orcinol reagent reacts with neuraminic acid to produce a green color having an absorption spectrum with a maximum at 580 mμ (8). With the unknown substance, this reagent formed a dark green color whose spectrum showed a maximum at 660 mμ. As a further test, the unidentified substance and neuraminic acid were both oxidized with periodate and reacted with thiobarbituric acid according to the procedure of Warren (9). The absorption spectra of the pink colored reaction mixtures were taken. That of neuraminic acid had a single maximum at 548 mμ, but that of the unidentified substance showed two maxima, one at 528 mμ and one at 450 mμ. The maximum at 450 mμ could be due to

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either formic or glyoxylic acid from the periodate oxidation. The 528 mμ peak is characteristic of 2-deoxyribose, but a Kiliani test for deoxy sugars was negative. Work is still continuing in attempts to identify this substance, which, because of the results of the above tests, does not appear to be neuraminic acid or 2-carboxypyrrole.

SE-Sephadex C-25 medium (Pharmacia Fine Chemicals, Inc.) was used in attempts to separate further the constituents in the flavor fraction. The resin, as received, was treated in 2 N sodium hydroxide by stirring briefly, then washed with distilled water until neutral. A 2.5 X 40 cm. column was poured, and the column equilibrated by passing 2 liters of 0.002 N hydrochloric acid through it. 200 mg. of the freeze dried diffusate was dissolved in 10 ml. of 0.002 N acid and applied to the column. The column was then eluted with the same strength HCl. 3 ml. fractions were collected at a flow rate of approximately 3 ml. per minute. Fractions were again read at 280 mμ. and readings recorded on graph paper. A typical plot of the curve is presented in Figure 2. Individual fractions were freeze-dried in the collection tubes and tested individually for the broiled steak odor. This was done by placing a small amount of the material from each tube on a cover slip on a Fisher melting point apparatus held at 145° C. temperature. Several runs were made, and in all cases, the strongest "broiled steak" odor was found in the fractions represented by 3 fractions on either side of the lowest point of the valley between the two peaks depicted in Figure 2. (See area marked by arrows).

The fractions, from the SE-Sephadex C-25 run, containing the "broiled steak" odor were examined by thin layer chromatography using silica gel G according to Stahl. 200 X 200 mm. plates were used. These were spotted with 15 individual spots and run in butanol-acetic acid-water 120:30:50 v/v. The dried plates were sprayed sectionally with ninhydrin (0.2% in acetone) for amino acids and peptides, aniline-diphenylamine reagent (10) for sugars, and ammonium molybdate reagent followed by exposure to U.V. light, for phosphates (10). Results are depicted in Figure 3. In comparing the results of these chromatograms with those from TLC plates of the fraction from Sephadex G-25 it was interesting to note the apparent decrease in the amount of ninhydrin positive material and the apparent large increase in carbohydrate material. Of further interest was the multiplicity of phosphate positive spots, since the carbohydrate material had been previously identified as glucose, and an experiment with paper chromatography was performed to determine whether any of the glucose was present as a phosphate ester. The system of Bieleski and Young (11) was used. Sheets of Whatmann #1 chromatographic grade filter paper were washed with 0.1 M borate buffer pH 9.5 and dried overnight. Duplicate sheets were spotted with glucose-1-phosphate, glucose-6-phosphate, glucose, inorganic phosphate (dibasic sodium phosphate) and the Sephadex fraction. Chromatograms were developed in 95% Ethanol / 0.1 M, pH 9.5 borate buffer (67/33 v:v). One chromatogram was checked for phosphate with

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ammonium molybdate reagent and the other with aniline-diphenylamine for sugars. R_f values were as follows:

		R_f with Phosphate Reagent	R_f with Sugar Reagent
G-1-P		.31	.29
G-6-P		.18	.14
Glucose			.40
P_i	Streaked	.28	
Fraction Seph.		.15	.40

These results indicate that the sugar present is free glucose and not one of its phosphate esters, since the sugar spot matched that of the control and the phosphate positive spot was considerably below that of the sugar. Of further interest is the observation that in this system only one phosphate spot was evident as compared to a number of phosphate spots in the butanol-acetic acid-water system on the TLC plates. Work is continuing in efforts to identify the phosphate material.

The observation from the TLC experiment that the ninhydrin positive material had decreased considerably, was of some concern since there seems to be a general agreement in the literature that a Maillard condensation between amino acids and sugars is involved in flavor formation. To determine whether a real loss of ninhydrin positive material had occurred, an amino acid analysis was run on the acid hydrolyzate of the SE SEPHADEX C-25 fraction. The hydrolyzate was prepared by heating the fraction with constant boiling hydrochloric acid, in a sealed combustion tube at 100° C. for 16 hrs. Results of this analysis are shown in Table 1, and show a definite loss of amino acids, compared to those obtained previously. If the Maillard condensation is involved in flavor production, it is difficult at the moment to reconcile the apparent increase in the "broiled steak" odor with such a drastic decrease in amino acid content of the flavor fraction. Considerably more information will have to be obtained before any final conclusions can be drawn.

(continued)

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Figure 1

Elution Diagram of Dialysate from Water Extract of Beef Muscle from Sephadex G-25 Column.

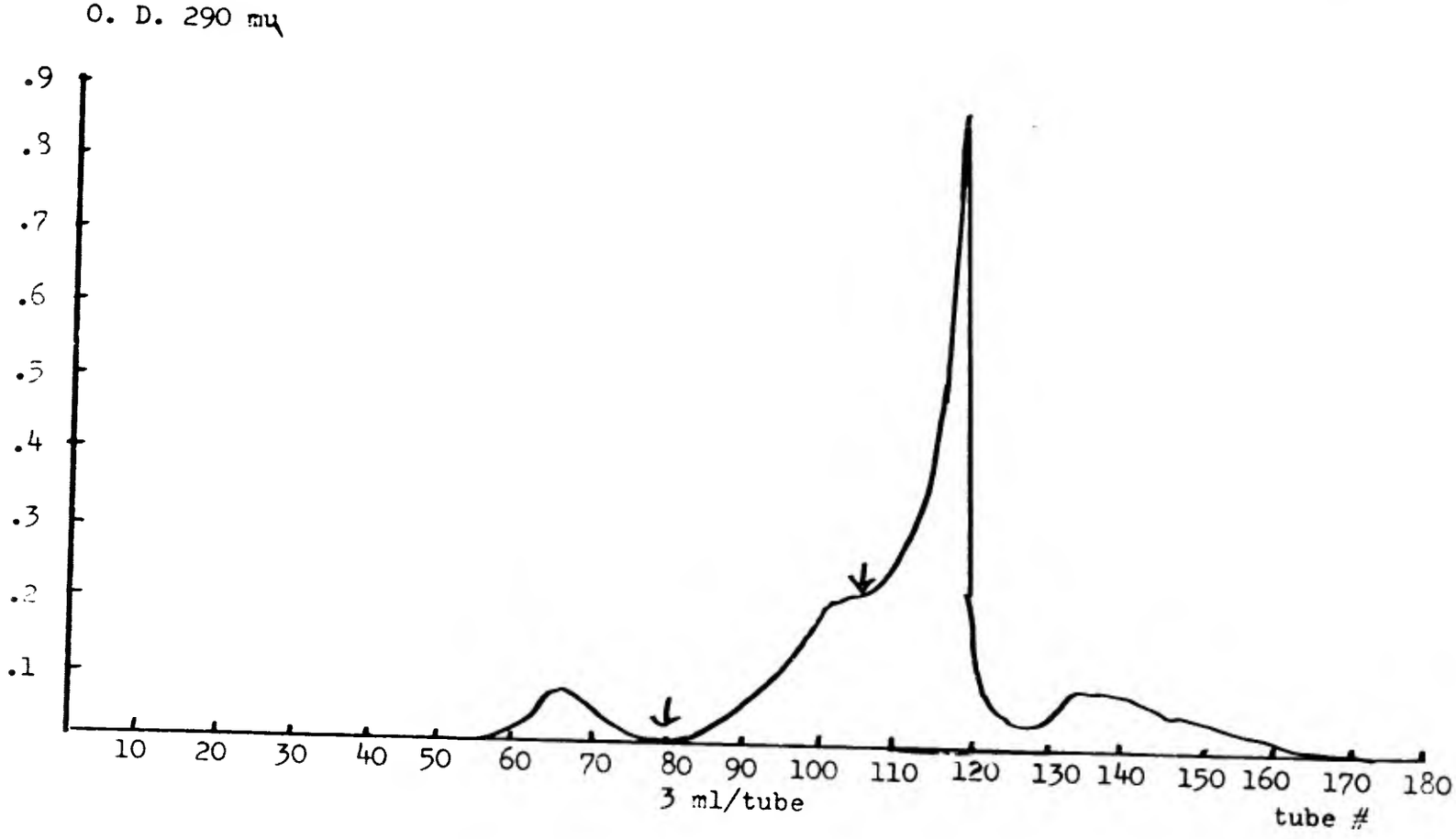


Figure 2
ELUTION DIAGRAM OF DIFFUSATE
FROM SE-SEPHADEX C-25 (Medium)
with 0.002N hydrochloric acid.

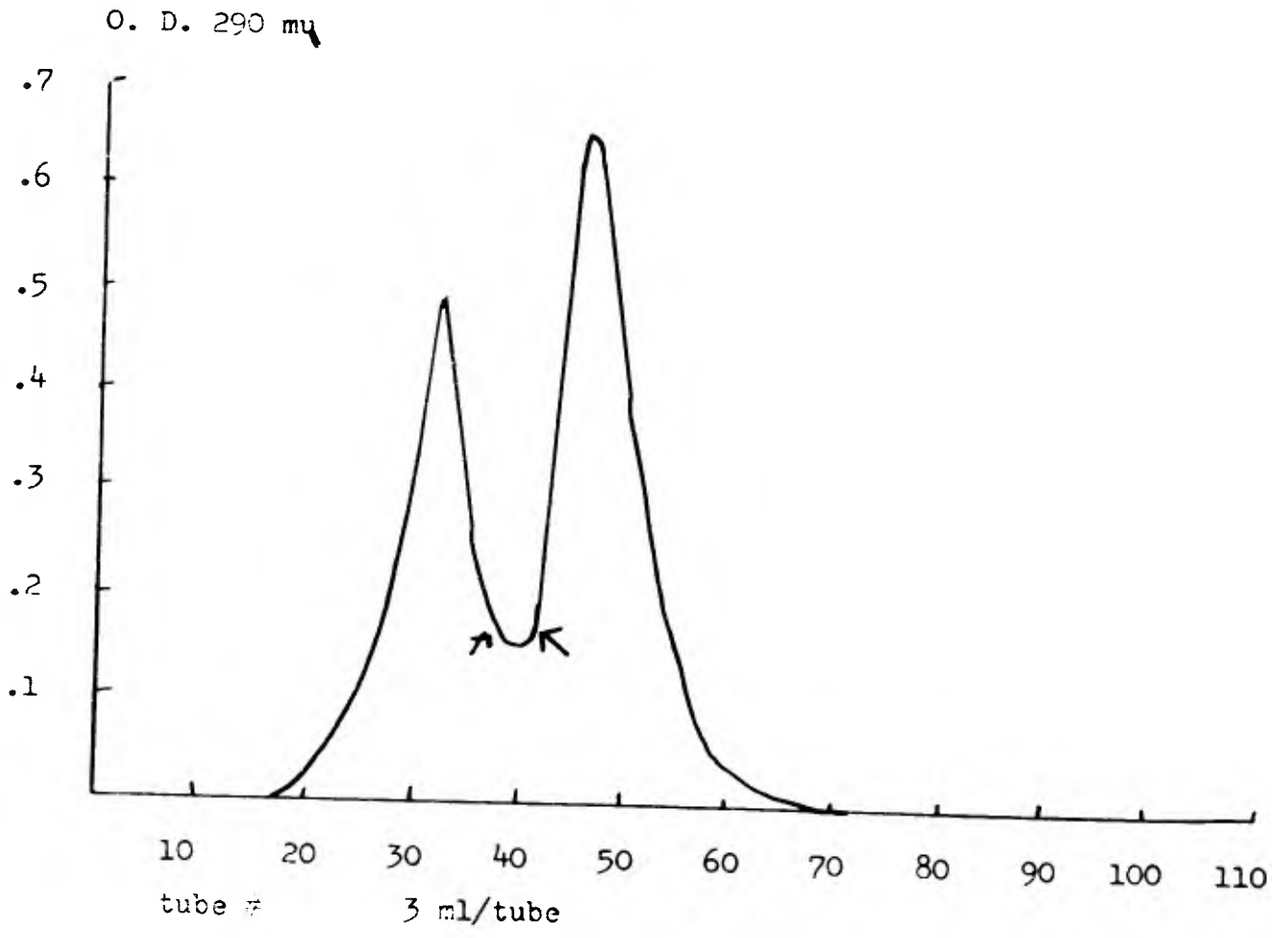
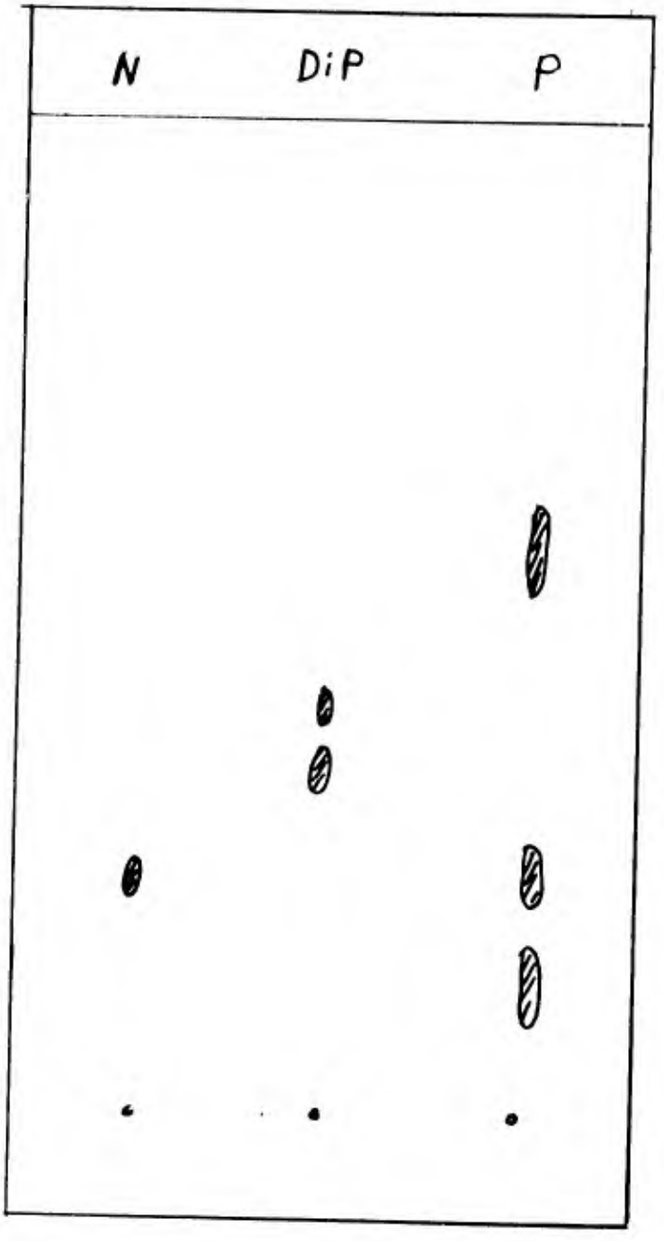


Figure 3
THIN LAYER CHROMATOGRAM
OF FRACTION FROM SE-SEPHADEX C-25 COLUMN.



N = NINHYDRIN
DIP = ANILINE-DIPHENYLAMINE REAGENT
P = AMMONIUM MOLYBDATE REAGENT

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Table 1

Amino Acid Analysis of
SE-Sephadex C-25 Fraction

	μ moles
Levulinic acid	1286
Aspartic acid	0.40
Threonine	0.06
Serine	0.03
Glutamic Acid	0.54
Proline	trace
glycine	0.84
alanine	trace
valine	"
isoleucine	"
leucine	"