DETERMINATION OF LYSINE IN WHEATS BY EXTRACTION, DINITROPHENYLATION AND THIN-LAYER CHROMATOGRAPHY

by 5791

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INTRODUCTION

A great many people in the world are underfed and malnourished. Because of their low cost and high yield, cereals are a major food staple accounting for two-thirds of the average per capita calorie intake in the Far East and over half of the calorie value of the diet in North Africa, West Asia and East Europe. Cereals are also relied upon as the major source of protein. Rice, wheat and corn are the major cereals and all are limited in certain essential amino acids, especially lysine. The lack of high quality protein in human diet is a major problem throughout the world (1).

Lysine is one of the essential amino acids for man and non-ruminant animals, and is known to be the most limiting amino acid in the protein of wheat. It is generally accepted that lysine in wheat flour can become nutritionally unavailable during storage. If the relative humidity is high (70%), or the moisture content of the sample exceeds certain limits, the Maillard reaction may take place at temperatures as low as 30-40°C. (2). That reaction results in the formation of enzyme-resisting linkages between free ε-amino group of lysine and the reducing group of carbohydrate. The digestive process of animals can not split such a linkage; however, the linkage may be quite labile to acid hydrolysis of the protein during amino acid analysis. Therefore, amino acid analysis give no information on the availability of lysine (3).

Animal feeding tests provide the best estimate of the availability of lysine, but the tests are slow and expensive. Thus, a faster, cheaper, and reliable chemical procedure is desirable for estimating the availability of lysine.

One of the simplest procedures (4) is reaction of 1-fluoro-2,4-dinitrobenzene (FDNB) with protein foodstuff. The bound ε-NH_2 group will not react with FDNB, while the free ε-NH_2 group will react with FDNB. The amount of lysine which had a free ε-NH_2 group and reacted with FDNB in fish and meat products was correlated well in assays with chicks when these products were used as supplement to basal diet (5). It is assumed that available lysine could be used as a measure of the quality of protein. Most of the FDNB chemical methods (3,4,6,7,8,9), among which the Carpenter method is well known, are derived from the method of Sanger (10).

The Carpenter (4) method has some minor limitations. In particular, it measures hydroxy-lysine and ornithine as lysine. Also, this method faces considerable difficulties when applied to cereal grains which are rich in carbohydrate, because the reaction of FDNB with carbohydrate decomposition products formed extraneous colored materials which interfered with colorimetry. Matheson (3,11,12) improved the method by isolating ε-DNP-lysine by column chromatography and determining the ε-DNP-lysine spectrophotometrically.

The purpose of this study was to apply dinitrophenylation to extracted wheat proteins to reduce the carbohydrate interference, to apply thin-layer chromatography to separate ε-DNP-lysine from other DNP-amino acids, to measure the ε-DNP-lysine directly on the TLC chromatograms by a Densitometer. All of these operations could result in simpler and a more sensitive method for the determination of available lysine in wheat.

REVIEW OF LITERATURE

Proteins are the only source of certain amino acids required by man and other animals. In nutrition, the quality of protein depends on its amino acid content and the availability of the amino acids to metabolic functions.

Cereals supply calories and protein in human food and livestock feed. Common cereal grains are limited in some amino acids essential in the human diet. Recently, favorable reports have shown the value of amino acid supplementation of basic grains as human food, such as proper supplementation of wheat with lysine, rice with lysine and threonine, and corn with lysine and tryptophan (13-16). Therefore, amino acid fortification can be considered as a means for improving protein quality to combat protein malnutrition.

Feeding experiments are considered the best way to estimate the protein nutritive value. The rat growth test for the evaluation of human diets has been used for a long time, since the rat appears to react in a comparable manner to the human being when meat is added to the diet (17).

The protein analysis of food provides an accepted mean of estimating the protein content. The Kjeldahl method is extensively used to measure the total nitrogen of food. Nitrogen-to-protein conversion factors of cereals and oilseeds range from 5.26 to 5.76 (18). Usually 5.7 for wheat and gluten, 6.25 for egg, meat and most feedstuffs containing vegetable proteins, and 6.29 to 6.49 for dairy products.

There are several ways to evaluate protein nutritive values such as net protein utilization (NPU), gross protein value (GPV), protein

efficiency ratio (PER), biological value (BV), net protein retention (NPR), and many others.

The availability of essential amino acids can be studied by in vivo and in vitro procedures. There are animal feeding trials in in vivo, and microbiological, enzymatic, and chemical procedures in in vitro. The purpose of the present study was to investigate the chemical procedures for determining the availability of lysine.

Lysine becomes unavailable nutritionally most easily of the known amino acids. An example was shown by Clegg and Dean (19) in their balance studies on peanut biscuit in the treatment of Kwashiokor. The biscuit made of peanut, corn, sugar, wheat flour, cottonseed oil and dried skim milk was baked and then hammer-milled into a fine powder. The diet did not have the expected nutritional value when it was compared with the diet having the same formula except for the addition of skim milk after the rest of the ingredients had been baked and milled. The lower nutritional value was due to the loss of available lysine by the reaction of milk lactose with the ε-NH_2 group of lysine during baking (20).

The mechanism of amino acid loss was studied by autoclaving soybean protein with sucrose for 4 hr. at 15 lb. pressure (21). Total and available amino acid contents of the protein were determined by microbiological assay after either acid or enzymatic hydrolysis. About 40% of lysine were found unavailable, presumably by a reaction of the free amino group of lysine with sucrose to form enzyme-resisting linkages.

Lea and Hannan (2) studied the interaction of casein and glucose in the dry state at pH 6.3, 70% relative humidity, and 37°C. The most rapid reaction occurred between glucose and the free amino groups of the protein, consisting mainly of the ε-amino groups of the lysine residues together

with a few terminal α-amino groups. The mechanism of the reaction is the interaction of aldehyde groups of reducing sugars with free amino group in protein to build an enzyme-resisting linkage by the Maillard (browning) reaction.

Gossypol, a polyphenolic pigment present in cottonseed, was found to react with the free ε-amino groups of lysine in cottonseed globulin. The availability of lysine, as determined by rat-feeding tests, was reduced when cottonseed meal was allowed to react with gossypol. Gossypol also reacted with the free ε-amino group of lysine in bovine serum albumin, egg albumin and β-lactoglobulin in addition to cottonseed globulin (22).

Mauron and Mottu (23) showed that the relationship between in vitro lysine availability and protein deficiency is essentially linear. In vitro enzymic hydrolysis of proteins, showed a good correlation with in vivo protein evaluation, using integrated indices based on the determination of the enzymic liberation of all essential amino acids. Their in vitro method of measuring lysine deterioration was very satisfactory in evaluation of the protein quality of dry milk powders.

The availability of lysine in all kinds of processed food, feedstuffs, dairy products and cereal grains has been studied by many investigators. The most commonly used method of measuring the available lysine is based on the Sanger (10) FDNB method. At room temperature, the ethanolic solution of 1-fluoro-2,4-dinitrobenzene (FDNB) reacts with protein at neutral to alkaline pH. The dinitrophenylation takes place in the free amino group (including the N-terminal amino group of protein and ε-amino group of lysine), the imidazole group of histidine, the phenolic-hydroxy group of tyrosine and the thiol group of cysteine. The dinitrophenylated (DNP-)

protein can then be hydrolyzed into the corresponding yellow-color DNP-amino acids. When DNP-amino acids are partitioned between ether and 1 N HCl, they divide into two groups (24). Most DNP-amino acids are ether soluble, including, α,ε-di-DNP-lysine. Among the water-soluble DNP compounds are: cysteic acid, citrulline, taurine, δ-ornithine, ε-hydroxylysine, ε-lysine, α-arginine, homoarginine, di-histidine, S-cysteine, and almost colorless compounds such as O-tyrosine and N^{im}-histidine.

Methods of assessing the available lysine based on the FDNB method have been sought by many workers. A simple procedure was proposed by Carpenter and Ellinger (6), and subsequently improved by Bruno and Carpenter (25), and by Carpenter (4). In the modified method of Carpenter (4), the protein foodstuff is dinitrophenylated and then hydrolyzed; a portion of the hydrolyzated is extracted with ether and the absorbance at 435 nm. of the aqueous phase is measured directly. Another portion is treated with methoxycarbonyl chloride converting ε-DNP-lysine to the ether-soluble ε-DNP-α-methoxycarbonyl derivative, which is then extracted. The absorbance at 435 nm. of the remaining aqueous phase is measured and the difference between the direct reading and the reading on the treated sample is taken as measuring the ε-DNP-lysine (available lysine). This new procedure is known as "Carpenter's FDNB procedure" which is used widely for determining the available lysine in foods and feeds. In this method, DNP-arginine was completely separated from ε-DNP-lysine because it does not react with methoxycarbonyl chloride. The N^{im}-DNP-histidine, if present in the aqueous phase, also reacts with methoxycarbonyl chloride to form an ether-soluble yellow derivative, thus interfering with the ε-DNP-lysine determination. Another limitation is

that hydroxylysine and ornithine are measured as lysine (3). Results with that method have been found to correlate well with the gross protein value (GPV) obtained by chick feeding tests.

ε-DNP-lysine is relatively resistant to acid hydrolysis. Porter and Sanger (26) obtained a 95% recovery of ε-DNP-lysine after 12 hr. of hydrolysis with 5.7 N HCl; recovery of other DNP-amino acids ranged from 10 to 90%. Carpenter (4) found that the mean recovery of added ε-DNP-lysine during analysis of foods was 92%. Matheson (3,12) found that the mean recovery of pure ε-DNP-lysine after 24 hr. of hydrolysis at 110°C. with 5.6 N HCl under vacuum in a sealed tube was 96%. Practically no loss of pure ε-DNP-lysine was found when it was carried through the extraction and chromatography stages of the analysis.

Lyman and Thomas (9) found that the loss of ε-DNP-lysine during hydrolysis of samples rich in carbohydrate was due to the reaction of the lysine derivative with polymerized carbohydrate decomposition products. In addition, the reaction of excess FDNB reagent with carbohydrate decomposition products formed extraneous colored materials which interfered with colorimetry. The latter factor can be corrected by chromatographically isolating the ε-DNP-lysine prior colorimetry. The carbohydrate interference can be reduced by addition of dinitrophenol and excess FDNB (3,12,27) during dinitrophenylation and acid hydrolysis. After acid hydrolysis, the excess FDNB and the dinitrophenol can be removed by ether extraction.

The isolation of ε-DNP-lysine for quantitative analysis was generally performed by paper and column chromatography (3,7,8,9,12). Baliga et al. (7) reported an accurate method for determination of free lysine ε-amino groups in cottonseed meal. The finely ground cottonseed

meal was dinitrophenylated for 22 hr. Excess reagent was removed by ether extraction. The residue was suspended in 6 N HCl and hydrolyzed under reflux for 18 hr. The hydrolyzate was extracted with ether to remove ether-soluble DNP-amino acids and ε-DNP-lysine was separated from the other water-soluble DNP-amino acids by paper chromatography using Whatman No. 3 MM paper ($18\frac{1}{2}$ by $22\frac{1}{4}$ cm.), and a solvent of butanol-acetic acid-water (4:1:1, v./v./v.). The heavy band of ε-DNP-lysine (R_f value 0.6) was cut out and eluted by 1 N HCl. The resulting yellow ε-DNP-lysine was measured spectrophotometrically at 363 nm. A rat protein-repletion test was used to determine protein quality in the cottonseed meal. A positive relationship was found between the content of free ε-amino lysine and protein quality.

Rao et al. (8) developed a method for determining the available lysine in oilseed meal proteins. The defatted ground sample was dinitrophenylated, excess reagent was removed by ether extraction, the dry residue was hydrolyzed overnight under reflux, and an aliquot of the hydrolyzate was column chromatographied to separate ε-DNP-lysine. The eluting solvent was a mixture of methyl ethyl ketone-3 N HCl (1:3, v./v.). The resulting yellow ε-DNP-lysine solution was measured spectrophotometrically at 435 nm.

Matheson (3,11,12,24) improved the methods of Carpenter (4), Rao et al. (8), and Lyman and Thomas (9). A small amount of protein sample was used for dinitrophenylation; thus the amount of chemical reagents used was greatly reduced. After dinitrophenylation, the remaining FDNB and dinitrophenol were not removed by ether extraction; thus ε-DNP-lysine can be partly protected from destruction by FDNB and by dinitrophenol during acid hydrolysis in a sealed tube under vacuum. After the solvent

partition of the DNP-amino acids, the ε-DNP-lysine was separated from other water-soluble DNP-amino acids by column chromatography (12,24) using a stationary phase of tris-glycine buffer, pH 9.1, saturated with the mobile phase (a mixture of ethyl acetate and ethyl methyl ketone) and supported on Kieselguhr. The yellow band of ε-DNP-lysine (R_f value 0.35) was separated and measured spectrophotometrically at 350 nm. (a maximum) and at 400 nm. (on a shoulder). The amounts of ε-DNP-lysine determined at each wavelength must agree; if not, the ε-DNP-lysine was contaminated with light-absorbing impurities. Matheson's method (12) was, however, subject to carbohydrate interference.

The available lysine determination by the FDNB methods estimated only the non-N-terminal lysine in protein. N-terminal lysine became α,ε-di-DNP-lysine not ε-DNP-lysine and, therefore, was not estimated. The amount of di-DNP-lysine is small from most samples, unless free lysine has been added.

Okuyama and Satake (28) reported that 2,4,6-trinitrobenzene-sulfonic acid (TNBS) reacted with only primary amino groups of amino acids and peptides in an aqueous solution at pH 8 and at room temperature. No reaction occurs with the imidazole group of histidine, the quanidium group of arginine, or the hydroxyl group of tyrosine or threonine. The resulting TNP-peptides, as well as TNP-amino acids, had a maximum absorbance at 340 nm. The acid soluble TNP-amino acids (not extractable with ether); α-mono-TNP-cysteic acid, α-TNP-arginine, α-TNP-histidine and ε-mono-TNP-lysine could be separated by paper chromatography using a mixture of n-butanol-acetic acid-water (4:1:2, v./v./v.).

Satake et al. (29) reported that TNP-amino acids and TNP-peptides were not as stable as the corresponding DNP-derivative. They were

photo-susceptible and were decomposed by concentrated ammonia, as shown by the following equations:

Photolysis

$$\text{TNP-NH-CHR-COOH} \xrightarrow{h\nu} \text{TNP-NH}_2 + \text{R-CHO} + \text{CO}_2$$

Ammonolysis

$$\text{TNP-NH-CHR-COOH} \xrightarrow{NH_3} \text{TNP-NH}_2 + \text{NH}_2\text{-CHR-COOH}$$

Kotaki and Satake (30) have revealed that the TNP-NH linkage of α-amino acid is much less stable than the DNP-NH linkage; thus TNP-α-amino acids are broken into picric acid and the original amino acids by acid or alkali.

$$\text{TNP-NH-CHRCOOH} \xrightarrow{\text{acid or alkali}} \text{TNP-OH} + \text{NH}_2\text{-CHR-COOH}$$

Acid: 6 N HCl, 110°C., 8 hr.

Alkali: 1 N NaOH, room temperature, 3 hr.

Such a susceptibility of TNP-NH linkage would provide some advantages as well as some restrictions for the use of TNP method in protein chemistry. TNP-ε-NH linkage and TNP-δ-NH linkage were rather resistant to acid, and more than 60% were capable of remaining intact even after the prolonged heating for 24 hr. in 6 N HCl.

Habeeb (31) used TNBS for determination of the free amino group of proteins and found that the optimum pH for trinitrophenylation was at pH 8.5 for bovine serum albumin, human γ-globulin fraction II, and ovalbumin.

Mokrasch (32) modified the method of Satake et al. (29) to increase the sensitivity and selectivity. He observed that the absorption spectrum of the product of the reaction between TNBS and derived amines is not identical to the absorption spectrum of the corresponding purified TNP-derivative. Mixtures of amines, amino acids, and proteins can be

analyzed quantitatively for their constituents by selective extraction of the TNP-derivatives. Amines and most amino acids may be analyzed by the absorbance of the TNP-derivatives at 420 nm. Aromatic amino acids, proteins and TNP-derivatives extracted into organic solvents can be estimated by absorbance near 340 nm.

Kakade and Liener (33) in 1969 reported a method to determine the available lysine in protein foodstuffs. The protein sample was trinitrophenylated with TNBS reagent at 40°C., pH 8.5 for 2 hr. to form TNP-protein. The resulting TNP-protein was hydrolyzed by autoclaving at 120°C. (15-17 psi) for 1 hr. Ether-soluble TNP-amino acids, small TNP-peptides, excess TNBS, and picric acid (a by-product of decomposed TNBS) were removed by ether extraction. The ε-TNP-lysine in aqueous solution was measured colorimetrically at 346 nm. The Kakade and Liener's TNBS method (33) for determining available lysine is not very specific, it cannot differentiate arginine and histidine from lysine. Other amino acids and impurities can also affect the analytical result.

Some cereal proteins can be quantitatively extracted by 0.2% NaOH (34,35). The use of a protein extract for dinitrophenylation gives better results in estimating available lysine in cereal grain due to the decreasing of the carbohydrate interference (9). Dinitrophenylation of denatured proteins is easier than that of native ones (36). Most of the protein samples should be denatured by grinding, protein extraction, and exposing to ethanol. The use of 60% ethanol as a solvent for dinitrophenylation of protein samples was found to help obtain a complete reaction (37).

Thin-layer chromatography (TLC) has become one of the most widely used modern analytical techniques in chemistry. A solvent system of n-propanol-34%

ammonium hydroxide (70:30, v./v.) can be used for the thin-layer chromatography of acid- and water-soluble DNP-amino acids. ε-DNP-lysine, DNP-arginine, DNP-cysteic acid, mono-DNP-cystine, α-DNP-histidine, di-DNP-histidine, and O-DNP-tyrosine can be separated by that system (38). The time required is about 2 hr. Stahl (38) listed the R_f values and means of differentiating the spots (Table I).

TABLE I. IDENTIFICATION OF ACID- AND WATER-SOLUBLE DNP-AMINO ACIDS BY TLC ON SILICA GEL G IN THE SYSTEM N-PROPANOL-34% AMMONIUM HYDROXIDE (70:30, V./V.). ASCENDING TECHNIQUE, SOLVENT MIGRATION 10 CM., 0.5-1.0 μG. SPOTTED.

DNP-amino acid	R_f[a]	Color	Absorbance (360 nm.)	Color with ninhydrin
Mono-DNP-cystine	0.29	yellow	+	brown
DNP-cysteic acid	0.29	yellow	+	yellow
α-DNP-arginine	0.43	yellow	+	yellow
ε-DNP-lysine	0.44	yellow	+	brown
O-DNP-tyrosine	0.49	colorless	+	violet
α-DNP-histidine	0.57	yellow	+	yellow
Di-DNP-histidine	0.65	yellow	+	yellow

[a]Average of the results of 6 separate measurements.

Although DNP-arginine and ε-DNP-lysine are not completely separated, each can be detected in the presence of the other by their different colors with the ninhydrin reaction. DNP-cysteic acid and mono-DNP-cystine should never occur together in practice. Theoretically, N^{im}-DNP-histidine belongs to the group of acid-soluble DNP-derivatives. Zahn and Pfannmuller (39) state that it cannot be detected in hydrolyzates of the corresponding DNP-peptides on account of its instability. Therefore, they have restricted their study to the α-DNP-and di-DNP-histidine derivatives. Excess acid must be removed after the sample is applied to the plate.

Densitometry provides sensitive and reproducible results for quantitative evaluation of thin-layer chromatograms, paper chromatograms, and electrophoresis patterns (40,41,42). The principle of densitometry is very similar to that of colorimetry. Each spot on the chromatoplate gives a peak of density values; the area under the peak is a function of size and density of the spot and is used for quantitative analysis (41).

EXPERIMENTAL

I. Materials

1. Samples.

1-1. Proteins.

Insulin: Crystalline (pfs) from Bovine Pancreas (NO. I-5500) Sigma Chemical Company, St. Louis, Mo.

Albumin, Egg: (Ovalbumin) Grade V. (NO. A-5503) Sigma Chemical Company.

α-Chymotrypsinogen A: Type II. (pfs) from Bovine Pancreas (NO. C-4879) Sigma Chemical Company.

Lysozyme: (Muraminidase) Grade I from Egg White (NO. L-6876) Sigma Chemical Company.

Catalase: Purified powder from Bovine Liver (NO. C-10) Sigma Chemical Company.

Cytochrome C: from Horse Heart (NO. 05100-1581) Mann Research Laboratories, Division of Becton-Dickinson and Company, New York, N.Y.

γ-Globulins: (Human) (NO.17200-457) Mann Research Lab., Division of Becton-Dickinson and Company.

Glutenin: Nutritional Biochemicals Corporation, Cleveland, Ohio.

1-2. Flour.

Red River 68 wheat flour (Semi-Dwarf Wheat) from World Seed Inc., North Dakota.

Sodium caseinate. (fine powder).
Fish flour.
Soy flour.
Chickpea flour. (Cicer arietinum).
Broad bean flour.
Supplied by Department of Grain Science and Industry, K.S.U., Manhattan, Kansas.

1-3. Wheats.

Wheat samples NO. 6, 7, 9, 10, 11, 12, 15, 16, 17, 18, 19, 20, 21, 22, 23, and 24 from Department of Agronomy, University of Nebraska, Lincoln, Nebraska.

2. Chemical Reagents.

All chemicals used in this study were reagent grade.

Sodium hydroxide: J. T. Baker Chemical Co., Phillipsburg, N.J.

Sodium bicarbonate: Allied Chemical, General Chemical Division, Morristown, N.J.

Ethanol (absolute): U.S. Industrial Chemicals Co., New York, N.Y.

1-Fluoro-2,4-dinitrobenzene (FDNB): Sigma Chemical Co.

Hydrochloric acid (Conc.): Allied Chemical.

Ether anhydrous (peroxide free): Allied Chemical.

n-Butanol: Fisher Scientific Co., Fair Lawn, N.J.

n-Propanol: Mallinckrodt Chemical Co., St. Louis, Mo.

Ammonium hydroxide: Mallinckrodt Chemical Co.

ε-DNP-lysine HCl: 1 μg./μl. 1 N HCl solution, (NO. D-0380) Sigma Chemical Co.

N-DNP-l-arginine: 1 μg./μl. 1 N HCl solution, Sigma Chemical Co.

O-DNP-tyrosine: 1 μg./μl. 1 N HCl solution, Nutritional Biochemicals Corporation, Cleveland, Ohio.

Silica Gel G for TLC acc. to Stahl: E. Merck Reagents Div. Brinkmann Instruments, Westbury, N.Y.

Ninhydrin: Ninhydrin solution for spraying (0.3 g. ninhydrin is dissolved in 100 ml. n-butanol, and 3 ml. acetic acid added), Nutritional Biochemicals Corporation.

Glacial acetic acid: Mallinckrodt Chemical Co.

2,4,6-Trinitrobenzenesulfonic acid (TNBS): 0.1% and 1.0% aqueous solution, Nutritional Biochemicals Corporation.

ε-TNP-l-lysine HCl: 1 μg./μl. 1 N HCl solution, Nutritional Biochemicals Corporation.

α-TNP-l-arginine: 1 μg./μl. 1 N HCl solution, Nutritional Biochemicals Corporation.

α-TNP-l-histidine H_2O: 1 μg./μl. 1 N HCl solution, Nutritional Biochemicals Corporation.

3. Instruments.

Analytical balance.

Wrist-action shaker: Model B B, Burrell Corp., Pittsburgh, Pa.

Automatic superspeed centrifuge: Model SS-3, Ivan Sorvall, Inc., Norwalk, Conn.

Shaker: Eberbach, Ann Arbor, Mich.

Electrically heated oven.

Test-tube mixer: (Super mixer) for thorough mixing in all sizes of test tube, Cole-Parmer, Chicago, Ill.

Rotary evaporator: Cole-Parmer Instrument & Equipment Co., Chicago, Ill.

Commercial spreader: (Stahl TLC spreader) C.A. Brinkmann and Co., Great Neck, N.Y.

Densitometer equipment: Photovolt Densitometer Model 530 with a Multiplier Photometer Model 520-A as a power source and a Variable-Response Recorder Model 42-A, from Photovolt Corporation, N.Y.

Compensating Polar Planimeter: for the accurate measurement of plane areas of any form, (Gelman Instrument Co., Germany) Keuffel & Esser Co., Kansas City.

Water bath with shaker: Precision Scientific Co., Chicago, Ill.

Autoclave: Operating pressure 15 psi, American Sterilizer Co., Erie, Pa.

Hammer mill: (Laboratory pulverizing mill) (0.022 inch dia. screen), Weber Bros & White Metal Works, Inc., Chicago, Ill.

II. Extraction of Wheat Protein with Alkali.

1. Testing the Procedure

An 0.2% NaOH solution has been reported as the best solvent to extract protein from cereal grain (34,35). The ratio of 2 g. flour vs. 50 ml. solvent was used, so that the protein in 1 ml. of the extract would contain 20 to 100 μg. non-N-terminal lysine for dinitrophenylation (12). Several procedures were tested using Red River 68 wheat flour. A 20 ml. sample of protein extract (equal to 0.8 g.

wheat flour protein) along with 1 g. flour sample were analyzed for total nitrogen. The results were used to calculate extraction efficiency. Proximate analysis (43) for protein was analyzed by AACC 46-12 Kjeldahl method and for moisture content was analyzed by AACC 44-19 air-oven method. The results showed that the wheat protein can be quantitatively extracted by the following procedure.

2. Procedure for Wheat Protein Extraction.

Two grams wheat flour was weighed in 50 ml. centrifuge tube, 20 ml. of 0.2% NaOH was added, and the tube was stoppered. The sample was shaken with wrist-action shaker for 1 hr. at room temperature. The sample was then centrifuged for 20 min. at 9000 r.p.m. (r = 4.25 in., C.F. = 9750 G), and the supernatant solution was collected. The residue was repeatedly extracted by an additional 20 ml. of 0.2% NaOH for 1 hr., and the sample was centrifuged. Finally, the residue was repeatedly extracted by additional 10 ml. of 0.2% NaOH for 30 min., and the sample was centrifuged. The combined extract was thoroughly mixed before use.

3. Extraction Efficiency

The average extractable nitrogen of four determinations (97.51%, 97.59%, 97.73% and 97.88%) was 97.70% $\pm$ 0.15%.

III. Modified FDNB Method.

1. Dinitrophenylation, Acid Hydrolysis, and Solvent Partition of DNP-Amino Acids.

1-1. For Protein Samples.

Matheson's (12) second procedure (sealed vacuum method) was followed. The combined butanol extracts were evaporated to dryness, and dissolved in 0.5 ml of 1 N HCl for adjusting

the concentration of acid-soluble DNP-amino acids prior to thin-layer chromatography.

1-2. For Wheat Flour and Wheat Samples.

One ml. of the protein extract, 1.5 ml. of ethanol and 20 μl. of FDNB were used for dinitrophenylation. The time required for dinitrophenylation was increased to 2 hr. The procedures for acid hydrolysis and solvent partition of DNP-amino acids were the same as in the case of protein samples, except a filtration was required for the hydrolyzate which contained a dark brown precipitate (humin).

2. Thin-layer Chromatography for Separating ε-DNP-L-Lysine.

Several solvents suitable for thin-layer chromatography of amino acids on silica gel G has been reported by Stahl (38.44). A solvent system of n-propanol-34% ammonium hydroxide (70:30, v./v.) was found best for one-dimensional ascending thin-layer chromatography of protein or wheat protein hydrolyzate containing ε-DNP-lysine, α-DNP-arginine, O-DNP-tyrosine, and other water-soluble DNP-amino acids. After a 15 cm. solvent migration on a 20 by 20 cm. TLC plate (about 2 hr. 40 min.), ε-DNP-lysine was completely separated from other water-soluble DNP-amino acids and some unknown materials. (Figs. 1 and 2; pictures were taken under U.V. light). Upon spraying with ninhydrin reagent, the ε-DNP-lysine color changed from yellow to brown.

2-1. Method.

Glass plates (20 by 20 cm.) were coated with a 0.5 mm. layer of silica gel G. The coated plates were air dried overnight before use.

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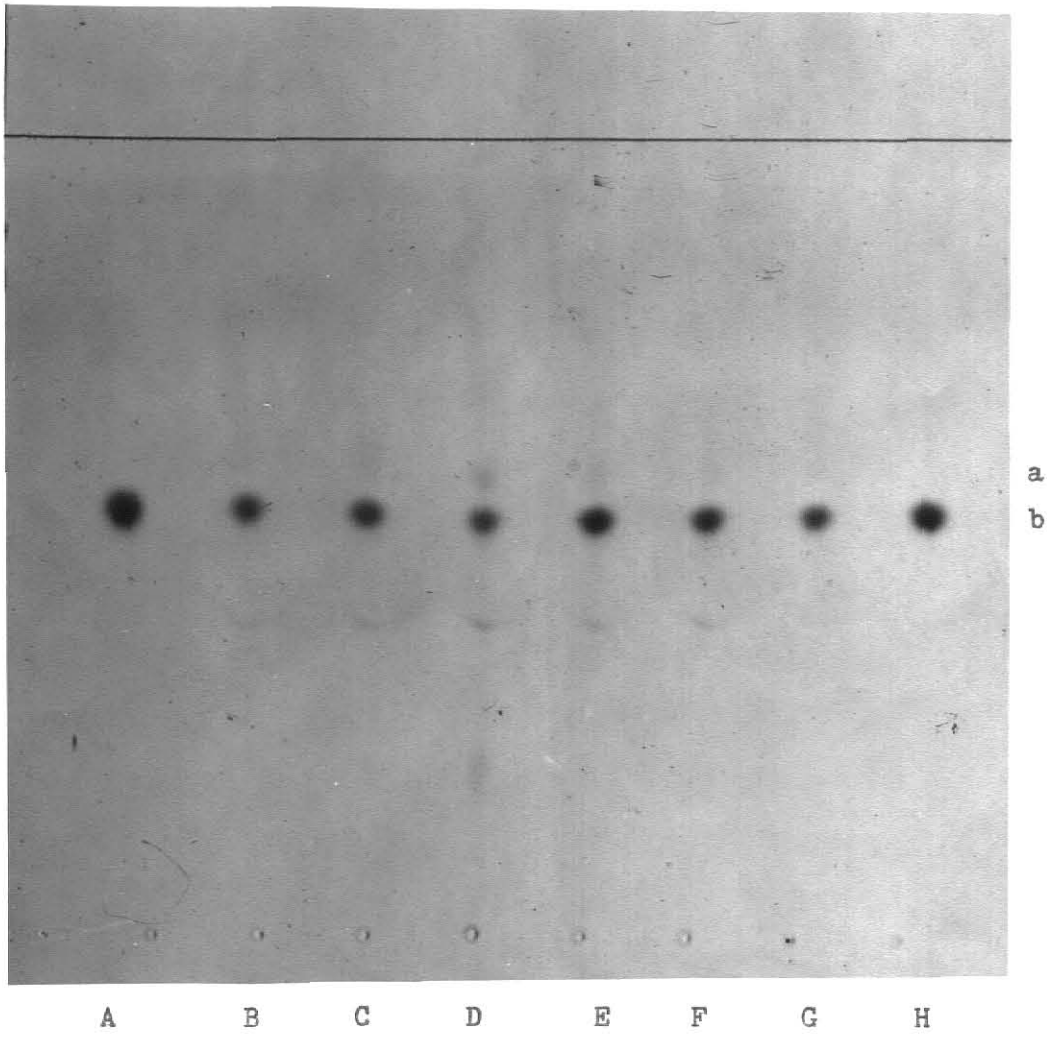

Fig. 1. Thin-layer chromatography of acid-soluble DNP-amino acids (not extractable with ether) from various protein samples.

A. Standard ε-DNP-l-lysine HCl
B. Ovalbumin (Albumin, Egg)
C. α-chymotrypsinogen A (Bovine Pancreas)
D. Insulin (Bovine pancreas)
E. Catalase (Bovine liver)
F. Lysozyme (Muramidase, Egg white)
G. Cytochrome C (Horse heart)
H. Standard ε-DNP-l-lysine HCl

a. O-DNP-tyrosine (colorless)
b. ε-DNP-lysine (yellow)

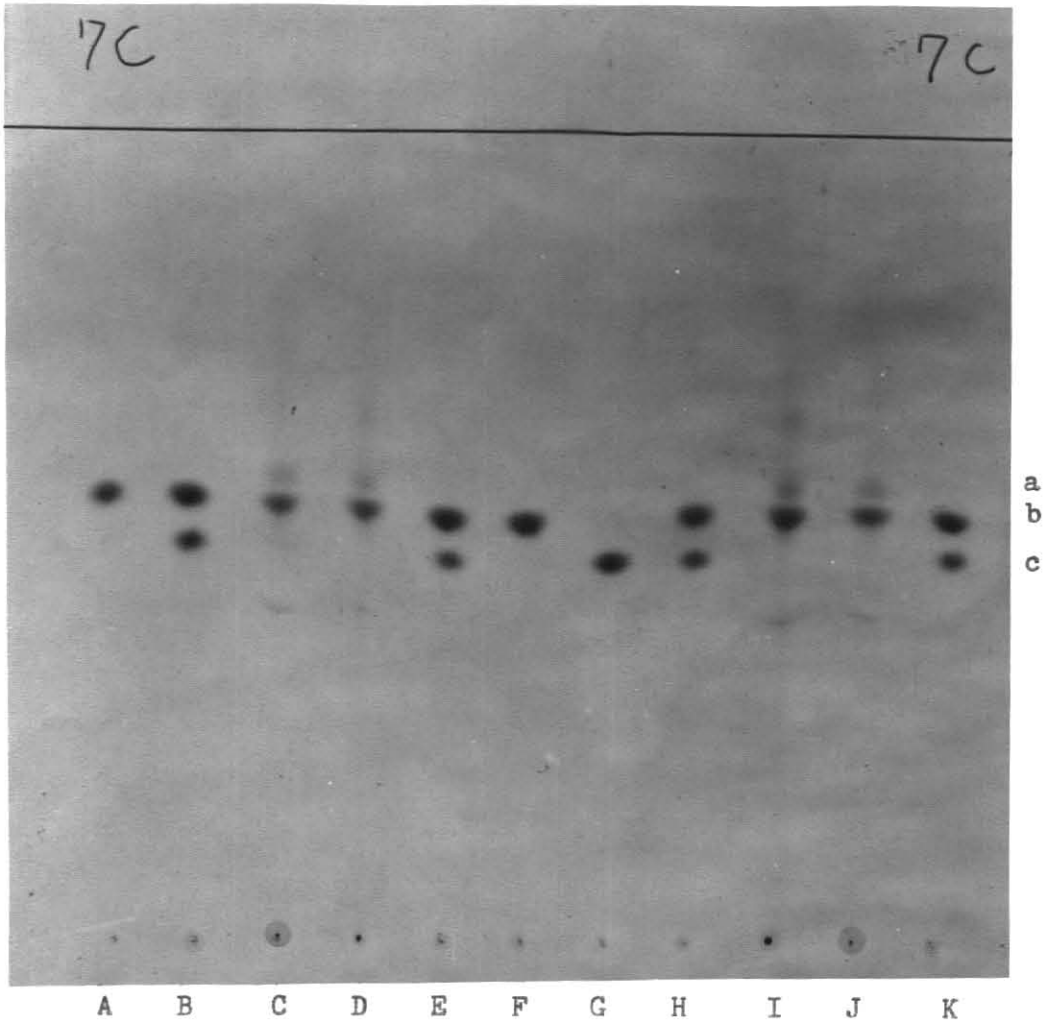

Fig. 2. Thin-layer chromatography of acid-soluble DNP-amino acids (not extractable with ether) from glutenin and wheat flour sample.

A. F.	Standard ε-DNP-l-lysine HCl
B. E. H. K.	Standard ε-DNP-l-lysine HCl + α-DNP-l-arginine
C. J.	Glutenin
D. I.	Red River 68 wheat flour
G.	Standard α-DNP-l-arginine
a.	O-DNP-tyrosine (colorless)
b.	ε-DNP-lysine (yellow)
c.	α-DNP-arginine (yellow)

Aliquots of standard solution (1 μg./μl. 1 N HCl ε-DNP-lysine HCl) and acid-soluble DNP-amino acids (prepared from proteins or wheat samples) were applied with a graduated lambda pipet in a small spot (3 mm. dia.) on the starting line 2 cm. from the bottom and separated by 1.5 cm. on the plate. Wet spots were dried with a hair dryer. The plate was developed ascendingly in a saturated chamber containing 110 ml. solvent of n-propanol-34% ammonium hydroxide (70:30, v./v.). After the solvent had migrated 15 cm., the plate was removed from the chamber and dried. The ε-DNP-lysine appeared as a yellow spot (about 3 to 5 mm. in dia.) with the same R_f value of standard ε-DNP-lysine (Figs. 1 and 2).

3. Quantitative Thin-layer Chromatography.

Standard ε-DNP-lysine HCl (1μg./μl.) was spotted in various concentrations to give 1, 2, 3, 4 and 5μg. per spot. The TLC chromatogram was developed and quantitatively measured the density of the spots by a Photovolt Densitometer Model 530 with a Multiplier Photometer Model 520-A as a power source, a Variable-Response Recorder Model 42-A, and a 420 nm. blue filter.

An 0.1 by 7.5 mm. upper slit was used. The dried chromatogram was mounted on the automatic drive, and the instrument was adjusted to 100% transmittance by selecting a background area. Each spot density was recorded continuously on a strip of chart paper. The peak area was integrated using a Gelman planimeter. Each curve was measured twice and the values averaged.

Generally, spots became larger, the higher they were allowed to migrate on the chromatoplate. A decrease in density was compensated

for by an increase in size. This relationship was illustrated by Blank et al. (41) on their experiment with tripalmitin. Their result showed that the peak area was essentially constant in the R_f range of 0.3 and 0.8.

Within a range of 1μg. to 5μg. of standard ε-DNP-lysine HCl, a plot of concentration vs. peak area (square inch) obtained from densitometry, gave a linear relationship. The width of the spots was smaller than the width of the upper slit (7.5 mm.); therefore, the spots were fully scanned. On different chromatograms, the linearity was the same, but the slope was different from one chromatogram to another (Tables II and III, and Fig. 3).

Within a range of 1μg. to 5μg. of ε-DNP-lysine HCl, the amount of ε-DNP-lysine (available lysine) in a sample can be calculated against a control with an known amount of standard ε-DNP-lysine.

4. Procedure for Modified FDNB Method.

After the preliminary experiments described in detail in the foregoing sections, a modified FDNB method employing thin-layer chromatography for quantitative estimation of available lysine in protein and wheat samples was developed. The modified method was summarized in the following procedure.

4-1. Extraction of Wheat Protein.

The procedure for wheat protein extraction was followed. A 20 ml. of protein extract along with 1 g. of flour sample were analyzed for total nitrogen by AACC 46-12 Kjeldahl method (43), and the results obtained were used to calculate the protein extraction efficiency.

TABLE II. THE DATA FOR STANDARD CURVE A. (MODIFIED FDNB METHOD)

Spot NO.	Concentration (μg.)	Peak Area[a] (sq. in.)	R_f	Width of spot (mm.)
1	1	0.29	0.53	3.0
2	2	0.53	0.53	3.0
3	3	0.76	0.53	4.0
4	4	1.00	0.52	4.5
5	5	1.21	0.52	5.0
6	1	0.30	0.52	3.0
7	2	0.53	0.52	3.0
8	3	0.76	0.52	4.0
9	4	0.98	0.52	4.5
10	5	1.23	0.52	5.0

[a]Fig. 3 was obtained by the average value of the peak area at the given concentrations.

TABLE III. THE DATA FOR STANDARD CURVE B. (MODIFIED FDNB METHOD)

Spot NO.	Concentration (μg.)	Peak Area[a] (sq. in.)	R_f	Width of spot (mm.)
1	1	0.29	0.55	3.0
2	2	0.51	0.54	3.0
3	3	0.74	0.53	4.0
4	4	0.95	0.51	4.5
5	5	1.16	0.51	5.0
6	1	0.30	0.51	3.0
7	2	0.51	0.51	3.5
8	3	0.72	0.52	4.0
9	4	0.94	0.52	4.5
10	5	1.16	0.52	5.0

[a]Fig. 3 was obtained by the average value of the peak area at the given concentrations.

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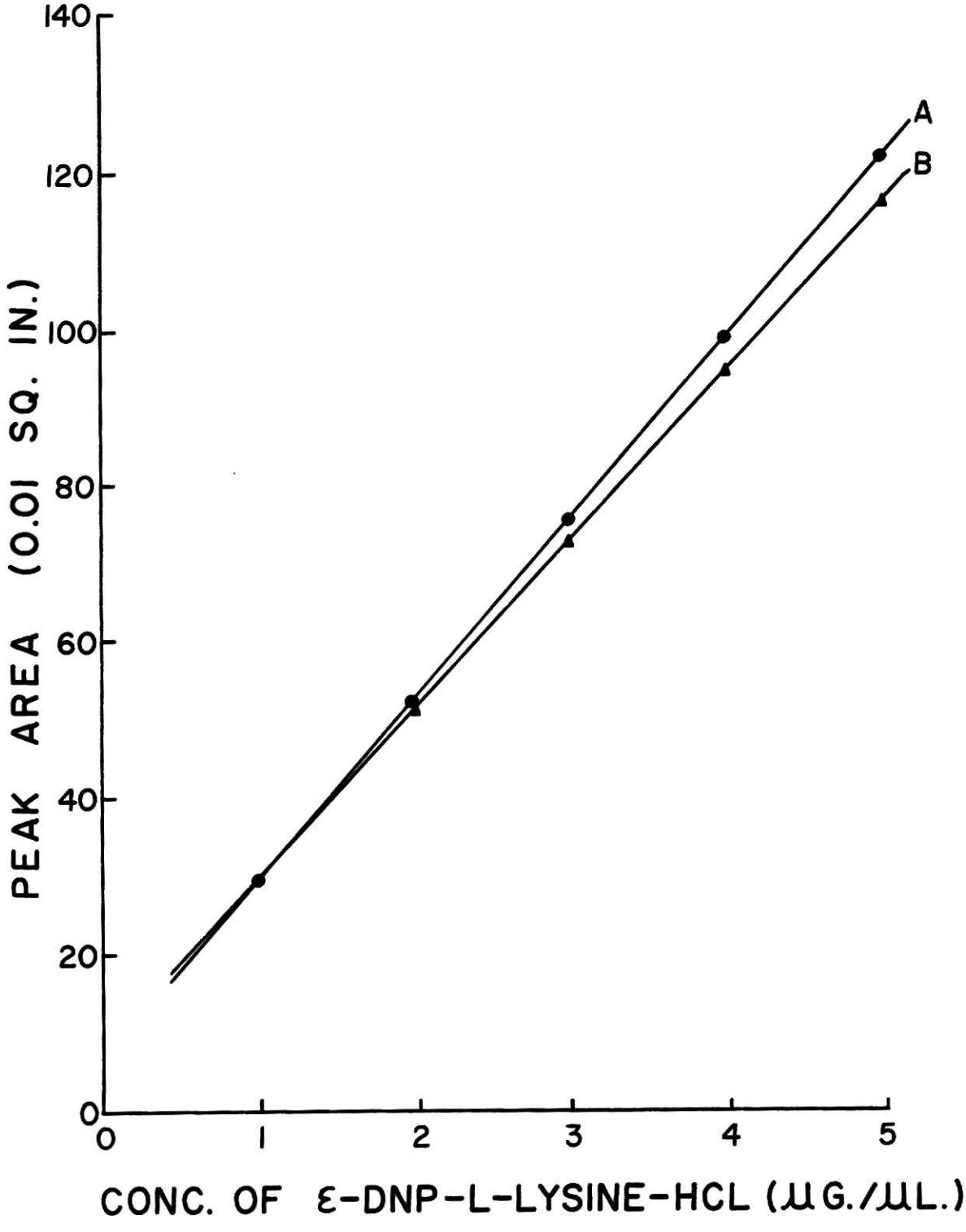

Fig. 3. Standard curve of modified FDNB method employing TLC for available lysine determination. A and B were in different TLC chromatograms.

4-2. Dinitrophenylation.

A sample of the protein or peptide, containing 20 to 100 μg. of non-N-terminal lysine, suspended in 0.5 ml. of 3% $NaHCO_3$, 0.75 ml. of ethanol and 10 μl. of FDNB were added. The suspension was shaken for 1 hr. at room temperature. In the case of wheat flour sample; 1 ml. of protein extract was added in the bottom of a glass tube, 1.5 ml. of ethanol and 20 μl. of FDNB were added. The suspension was shaken for 2 hr. at room temperature.

4-3. Acid Hydrolysis.

The dinitrophenylated mixture was evaporated to dryness by a water-pump, and dissolved in 1 ml. of 5.6 N HCl. The tube was then evacuated, sealed, and heated at 110°C. for 24 hr.

4-4. Solvent Partition of DNP-Amino Acids.

The hydrolyzate was evaporated to dryness under vacuum and transferred with 10 ml. of 0.75 N HCl to another test tube (200 by 19 mm.). In the case of the wheat samples, the hydrolyzate was evaporated to dryness under vacuum, transferred with 15 ml. of 0.75 N HCl and filtered through a funnel (Hirsch type, size F) into a long filtering test tube (200 by 19 mm.). The hydrolyzate was extracted with ether (3 times with 3 ml. portions), and then with n-butanol (4 times with 3 ml. lots). The ether extracts were discarded. The combined butanol extracts were evaporated to dryness in a rotary evaporator, and dissolved in 0.5 ml of 1 N HCl. The final yellow solution contained the acid-soluble DNP-amino acids from protein and wheat samples.

4-5. Thin-layer Chromatography.

TLC plates coated with 0.5 mm. layer of silica gel G were used. Triplicate spots of 3 μl. of standard ε-DNP-lysine HCl (1 μg./μl.) 1 N HCl solution and duplicate spots of 5 or 10 μl. of acid-soluble DNP-amino acids of four samples were applied on the same plate. The plates were developed ascendingly in a saturated chamber containing 110 ml. solvent of n-propanol-3% ammonium hydroxide (70:30, v./v.). After a 15 cm. solvent migration (about 2 hr. 40 min.) on the TLC plate, the plate was removed and dried.

4-6. Quantitative Thin-layer Chromatography.

The density of the ε-DNP-lysine spots was measured using a Photovolt Densitometer Model 530, with scanning stage and varicord recorder. The peak area was integrated using a Gelman planimeter. Each curve was measured twice and the values averaged. The amount of ε-DNP-lysine (available lysine) in a sample can be calculated against a control with an known amount of standard ε-DNP-lysine. The available lysine was expressed as g. lysine per 16 g. nitrogen for protein, and g. lysine per 100 g. sample for wheat and wheat flour.

4-7. Calculation of Available Lysine.

Data for the calculation of available lysine were presented in Table IV.

For protein sample, available lysine X (μg.) per 0.5 ml. of prepared protein solution was used for dinitrophenylation:

$$X\ (\mu g.) = \frac{3 \times Ap \times 500 \times 146.19}{As \times P \times 348.74} = 6.288 \times 10^2 \times \frac{Ap}{As \times P}$$

Available lysine Xp (g.) per 16 g nitrogen of protein sample:

$$Xp\ (g.) = 6.288 \times 10^2 \times \frac{Ap}{As \times P} \times \frac{1}{C} \times \frac{1}{10} = 62.88 \times \frac{Ap}{As \times P \times C}$$

Similarly, for wheat and wheat flour samples, available lysine Y (μg.) per 1.0 ml. of the protein-extract was used for dinitrophenylation:

$$Y\ (\mu g.) = \frac{3 \times Aw \times 500 \times 146.19}{As \times W \times 348.74} = 6.288 \times 10^2 \times \frac{Aw}{As \times W}$$

Available lysine Yw (g.) per 100 g. of wheat or wheat flour sample:

$$Yw\ (g.) = 6.288 \times 10^2 \times \frac{Aw}{As \times W} \times \frac{25}{1000} \times \frac{100}{E} \times \frac{1}{1000}$$

$$= 1.572 \times \frac{Aw}{As \times W \times E}$$

where all the values were as defined in Table IV.

TABLE IV. DATA FOR THE CALCULATION OF AVAILABLE LYSINE IN PROTEIN AND WHEAT SAMPLE (MODIFIED FDNB METHOD).

		Standard ε-DNP-lysine HCl	Protein Sample	Wheat Sample	L-Lysine
1.	Available lysine (μg.)	3	X	Y	
2.	Peak area (sq. in.)	As	Ap	Aw	
3.	Amount of final spotting (μl.)	3	P	W	
4.	Amount of acid-soluble DNP-amino acids (μl.)		500	500	
5.	Molecular weight	348.74			146.19
6.	Amount of solution for dinitrophenylation (ml.)		0.5	1.0	
7.	Amount of protein in 0.5 ml. protein solution (mg.)		C		
8.	% Wheat protein extraction			E	
9.	ml. of solution equal to 1 g. wheat sample			25	

5. Quantitative Estimation of Available Lysine in Protein and Wheat Samples.

Four protein samples (Insulin, Ovalbumin, α-Chymotrypsinogen A and Lysozyme) and sixteen wheat samples (NO. 6, 7, 9, 10, 11, 12, 15, 16, 17,

18, 19, 20, 21, 22, 23, and 24) were analyzed with the modified FDNB method. The results are shown in the Results and Discussion section.

IV. Modified TNBS Method.

1. Trinitrophenylation, Acid Hydrolysis, and Solvent Partition of TNP-Amino Acids.

Kakade and Liener (33) procedure was followed for both protein and wheat flour samples.

Both the hydrolyzate from protein sample and filtered hydrolyzate from wheat sample were extracted with ether (3 times with 5 ml. portions) then with n-butanol (4 times with 3 ml. lots). The combined butanol extracts were evaporated to dryness, and dissolved in 0.5 ml. of 1 N HCl for adjusting the concentration of acid-soluble TNP-amino acids prior to thin-layer chromatography.

2. Thin-layer Chromatography for Separating ε-TNP-L-Lysine.

A solvent system of n-butanol-glacial acetic acid-water (80:20:20, v./v./v.) was found best for one-dimensional ascending thin-layer chromatography of protein or wheat protein hydrolyzate containing ε-TNP-lysine, α-TNP-arginine, α-TNP-histidine and others. After a 15 cm. solvent migration on a TLC plate (about 3 hr.), ε-TNP-lysine was completely separated from other acid-soluble TNP-amino acids and some unknown materials. (Figs. 4, 5 and 6; pictures were taken under U.V. light). Upon spraying with ninhydrin reagent, the ε-TNP-lysine color changed from yellow to brown. Results of the experiment were presented in Table V.

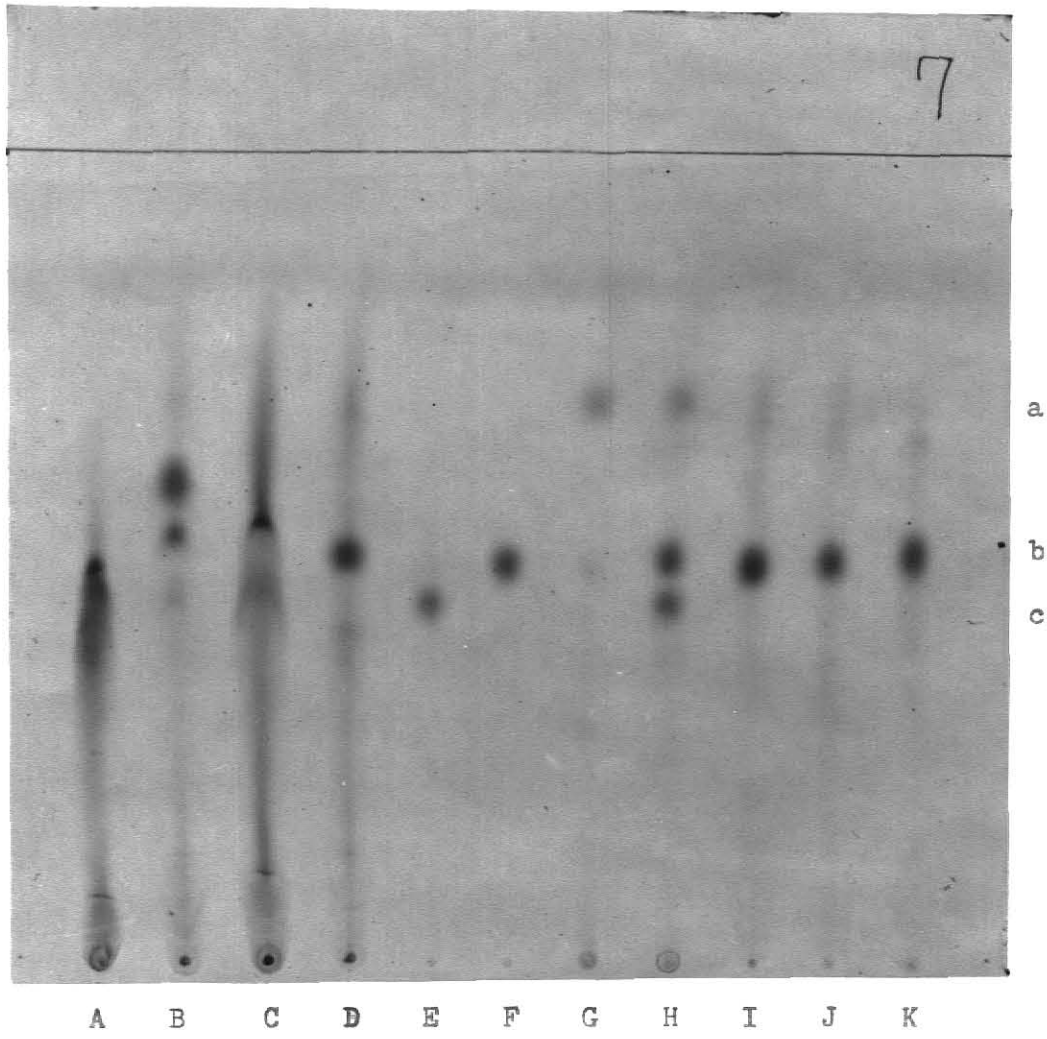

Fig. 4 Thin-layer chromatography of acid-soluble TNP-amino acids (not extractable with ether) from various proteins and flour samples.

A. Sodium caseinate
B. Fish flour
C. Soy flour
D. Red River 68 wheat flour
E. Standard α-TNP-histidine H_2O
F. Standard ε-TNP-lysine HCl
G. Standard α-TNP-arginine
H. Standard α-TNP-arginine + ε-TNP-lysine HCl + α-TNP-histidine H_2O
I. Catalase
J. Lysozyme
K. γ-globulins

a. α-TNP-arginine (yellow)
b. ε-TNP-lysine (yellow)
c. α-TNP-histidine (yellow)

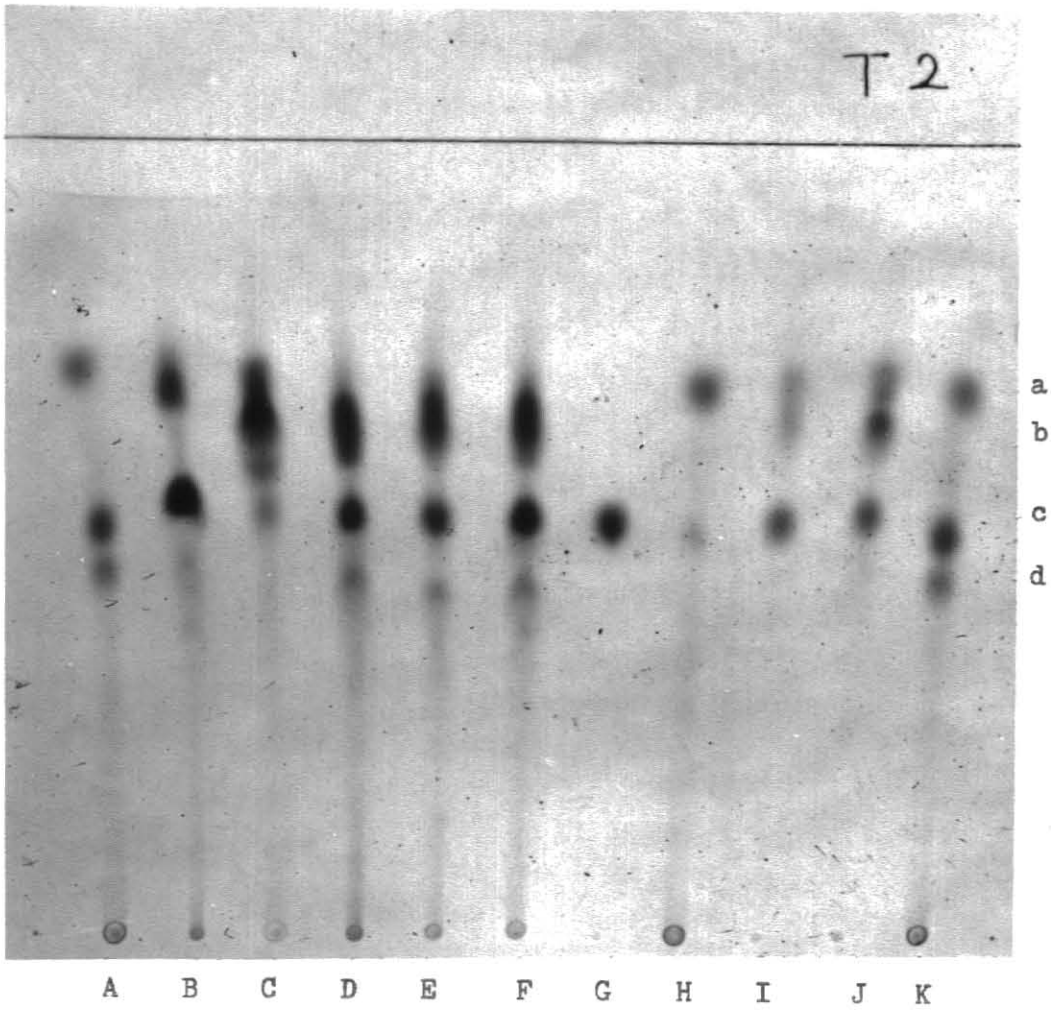

Fig. 5. Thin-layer chromatography of acid-soluble TNP-amino acids (not extractable with ether) from various proteins and flour samples.

A. K. Standard α-TNP-arginine + ε-TNP-lysine HCl + α-TNP-histidine H_2O
B. Sodium caseinate
C. Fish flour
D. Soy flour
E. Chickpea flour
F. Broad bean flour
G. Standard ε-TNP-lysine HCl
H. Standard α-TNP-arginine
I. Ovalbumin (Albumin, Egg)
J. Insulin (Bovine Pancreas)

a. α-TNP-arginine (yellow)
b. Unknown material (yellow)
c. ε-TNP-lysine (yellow)
d. α-TNP-histidine (yellow)

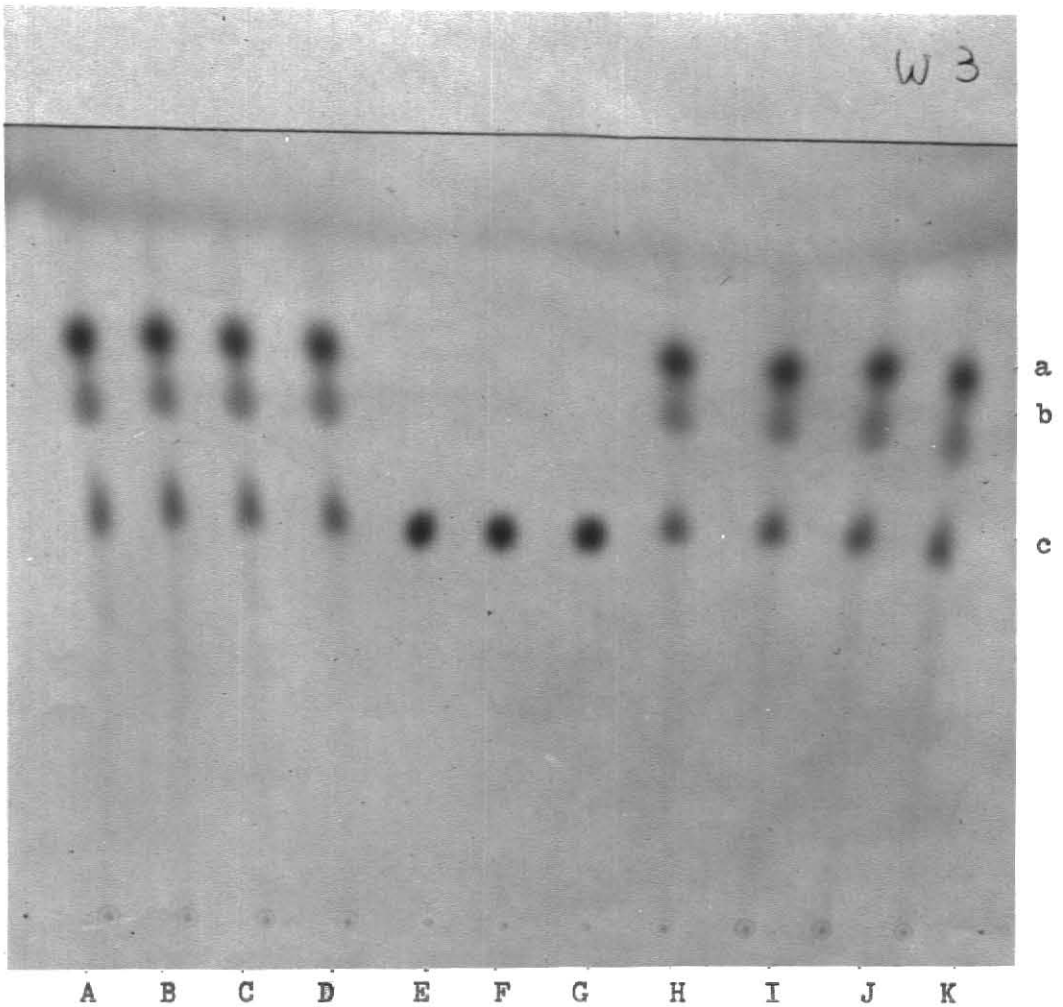

Fig. 6. Thin-layer chromatography of acid-soluble TNP-amino acids (not extractable with ether) from wheat samples.

A. B. H. I. Wheat NO. 23
C. D. J. K. Wheat NO. 24
E. F. G. Standard ε-TNP-lysine HCl

a. Unknown material (yellow)
b. Unknown material (yellow)
c. ε-TNP-lysine (yellow)

TABLE V. IDENTIFICATION OF ACID-SOLUBLE TNP-AMINO ACIDS (*NOT* EXTRACTABLE WITH ETHER) BY TLC ON SILICA GEL G, AND ALSO BY THE SYSTEM N-BUTANOL-GLACIAL ACETIC ACID-WATER (80:20:20, V./V./V.). ASCENDING TECHNIQUE, SOLVENT MIGRATION 15 CM., 3 μG. SPOTTED.

TNP-amino acid	R_f [a]	Color	Absorbance (360 nm.)	Color with ninhydrin
α-TNP-histidine	0.44	yellow	+	yellow
ε-TNP-lysine	0.51	yellow	+	brown
α-TNP-arginine	0.71	yellow	+	yellow

[a] Average of the results of 6 separate measurements.

2-1. Method.

The procedure was similar to that of modified FDNB method. A solvent of n-butanol-glacial acetic acid-water (80:20:20, v./v./v.) was used for one-dimensional ascending TLC. The ε-TNP-lysine appeared as a yellow spot (about 3 to 5 mm. in dia.) with the same R_f value of standard ε-TNP-l-lysine.

3. Quantitative Thin-layer Chromatography.

The procedure and principle were similar to that of modified FDNB method. A similar procedure was performed. Standard ε-TNP-lysine HCl (1 μg./μl.) was spotted in various amounts to give 2, 4, 6, 8 and 10 μg. per spot. The TLC chromatogram was developed and quantitatively measured by Photovolt Densitometer.

Within a range of 2 μg. to 10 μg. of standard ε-TNP-lysine HCl, a plot of concentration vs. peak area (square inch) obtained from densitometry, gave a linear relationship. On different chromatograms, the linearity was the same, but the slope was different from one chromatogram to another (Tables VI and VII, and Fig. 7).

TABLE VI. THE DATA FOR STANDARD CURVE A (MODIFIED TNBS METHOD)

Spot NO.	Concentration (μg.)	Peak Area[a] (sq. in.)	R_f	Width of spot (mm.)
1	2	0.26	0.52	0.55
2	4	0.45	0.52	0.65
3	6	0.67	0.52	0.65
4	8	0.87	0.51	0.70
5	10	1.08	0.51	0.70
6	2	0.25	0.51	0.60
7	4	0.45	0.51	0.65
8	6	0.66	0.50	0.70
9	8	0.89	0.50	0.70
10	10	1.09	0.49	0.70

[a]Fig. 7 was obtained by the average value of the peak area at the given concentrations.

TABLE VII. THE DATA FOR STANDARD CURVE B (MODIFIED TNBS METHOD)

Spot NO.	Concentration (μg.)	Peak Area[a] (sq. in.)	R_f	Width of spot (mm.)
1	2	0.26	0.53	0.50
2	4	0.45	0.52	0.60
3	6	0.60	0.52	0.60
4	8	0.78	0.51	0.65
5	10	0.97	0.50	0.70
6	2	0.26	0.50	0.50
7	4	0.43	0.51	0.55
8	6	0.62	0.51	0.65
9	8	0.78	0.52	0.65
10	10	0.95	0.52	0.65

[a]Fig. 7 was obtained by the average value of the peak area at the given concentrations.

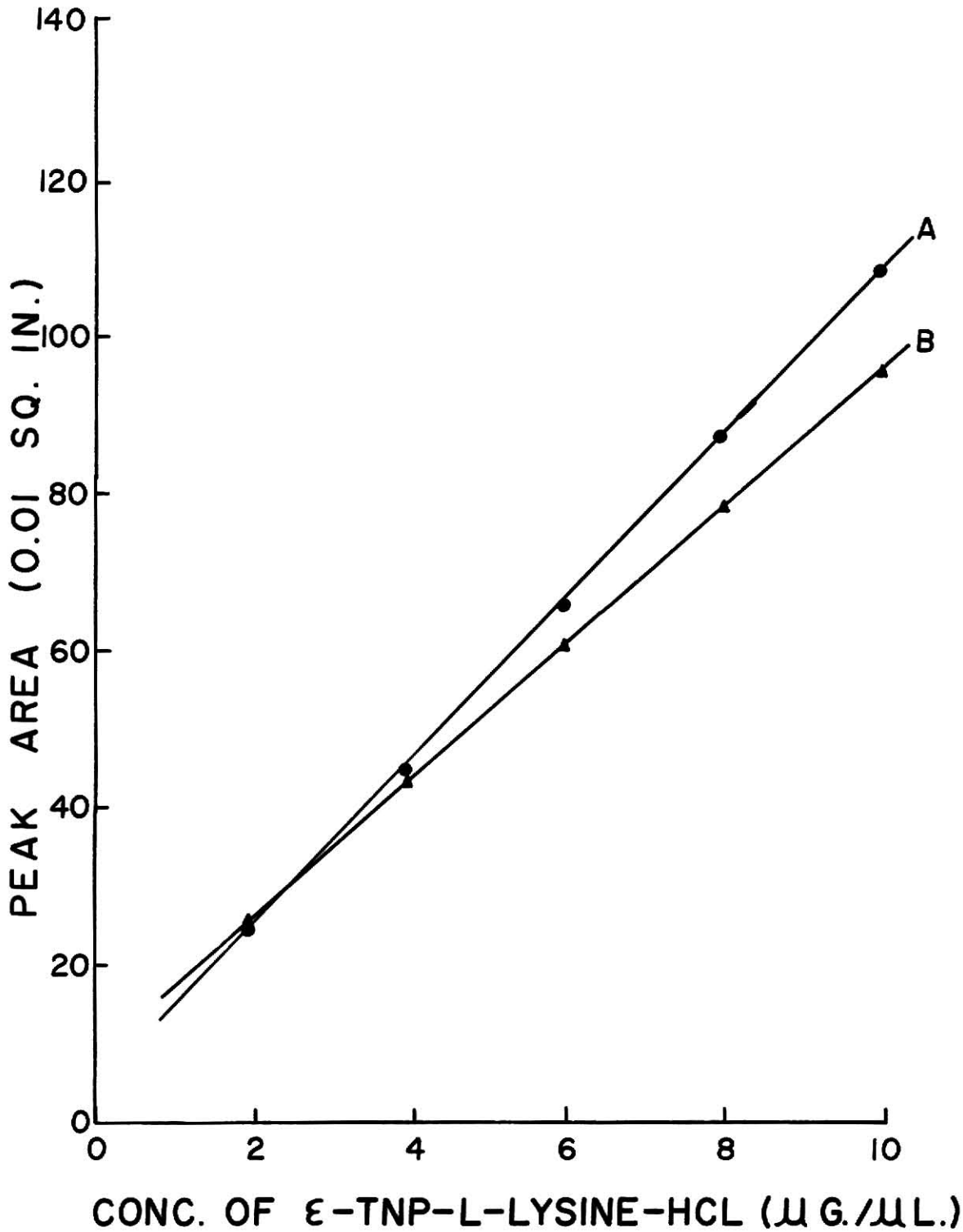

Fig. 7. Standard curve of modified TNBS method employing TLC for available lysine determination. A and B were in different TLC chromatograms.

Within a range of 2 μg. to 10 μg. of ε-TNP-lysine HCl, the amount of ε-TNP-lysine (available lysine) in a sample can be calculated against a control with an known amount of standard ε-TNP-lysine.

4. Procedure for Modified TNBS Method.

A modified TNBS method based on Kakade and Liener (33) method, but employing thin-layer chromatography for quantitative estimation of available lysine in protein was developed.

4-1. Trinitrophenylation.

One ml. of a freshly prepared 0.1% TNBS was added to 1.0 ml. of 4% $NaHCO_3$ solution (pH 8.5) containing 1 to 2 mg. of protein in a test tube (200 by 19 mm.). The reaction was allowed to proceed at 40°C. for 2 hr.

4-2. Acid Hydrolysis.

After trinitrophenylation, 3 ml. of concentrated HCl was added. An inverted glass vial was placed over the mouth of each tube, and the reaction mixture was hydrolyzed by autoclaving at 120°C. (15-17 psi) for 1 hr.

4-3. Solvent Partition of TNP-Amino Acids.

Five ml. of distilled water was added after the hydrolyzate had cooled to room temperature. The mixture was extracted with ether (3 times with 5 ml. portions) and then with n-butanol (4 times with 3 ml. lots). The ether extracts were discarded. The combined butanol extracts were evaporated to dryness in a rotary evaporator, and dissolved in 0.5 ml. of 1 N HCl. The final yellow solution contained the acid-soluble TNP-amino acids from protein.

4-4. Thin-layer Chromatography.

An 0.5 mm. layer of silica gel G coated TLC plates was used. Triplicate spots of 5 μl. of standard ε-TNP-lysine HCl (1 μg./μl.) 1 N HCl solution and duplicate spots of 5 or 10 μl. of acid-soluble TNP-amino acids of four samples were applied on the same plate. Using a one-dimensional ascending technique, the plates were developed with a solvent of n-butanol-glacial acetic acid-water (80:20:20, v./v./v.). After a 15 cm. solvent migration (about 3 hr.), the plate was removed and dried.

4-5. Quantitative Thin-layer Chromatography.

The density of the ε-TNP-lysine spots was measured using a Photovolt Densitometer Model 530, with scanning stage and varicord recorder. The peak area was measured with a Gelman planimeter. Each curve was measured twice and the values averaged. The amount of ε-TNP-lysine (available lysine) in a sample can be calculated against a control with an known amount of standard ε-TNP-lysine. The available lysine was expressed as g. lysine per 16 g. nitrogen for protein.

4-6. Calculation of Available Lysine.

Data for the calculation of available lysine were presented in Table VIII.

For protein sample, available lysine X (μg.) per 1.0 ml. of prepared protein solution was used for trinitrophenylation:

$$X\ (\mu g.) = \frac{3 \times Ap \times 500 \times 146.19}{As \times P \times 393.74} = 5.569 \times 10^2 \times \frac{Ap}{As \times P}$$

Available lysine Xp (g.) per 16 g. nitrogen of protein sample:

$$Xp\ (g.) = 5.569 \times 10^2 \times \frac{Ap}{As \times P} \times \frac{1}{C} \times \frac{1}{10} = 55.69 \times \frac{Ap}{As \times P \times C}$$

where all the values were as defined in Table VIII.

TABLE VIII. DATA FOR THE CALCULATION OF AVAILABLE LYSINE IN PROTEIN SAMPLE (TNBS METHOD)

	Standard ε-TNP-l-lysine HCl	Protein Sample	L-Lysine
1. Available lysine (μg.)	3	X	
2. Peak area (sq. in.)	As	Ap	
3. Amount of final spotting (μl.)	3	P	
4. Amount of acid-soluble TNP-amino acids (μl.)		500	
5. Molecular weight	393.74		146.19
6. Amount of solution for trinitrophenylation (ml.)		1.0	
7. Amount of protein in 1.0 ml. prepared protein solution (mg.)		C	

5. Quantitative Estimation of Available Lysine in Protein Samples.

Four protein samples (Insulin, Ovalbumin, α-Chymotrypsinogen A and Lysozyme) were analyzed with the modified TNBS method. The results are shown in the Results and Discussion section.

V. Results and Discussion.

The non-N-terminal lysine in four purified proteins was determined by both the modified FDNB method and the modified TNBS method. The results (average value of duplicate) are shown in Tables IX, X, XI and XII. To calculate recoveries it was assumed that the proteins were pure and that their non-N-terminal lysine content (g. lysine/16 g. nitrogen) were insulin, 2.57 (45), ovalbumin, 6.60 (37), α-chymotrypsinogen A, 7.59 (46), and lysozyme, 4.35 (47).

The available lysine in sixteen wheat samples was determined by the modified FDNB method, the per cent of available lysine in total lysine varied from 73.1% to 98.2% (Tables XIII and XIV). Total lysine was determined with Beckman Amino Acid Analyzer (Model 120C) after hydrolysis with 6 N HCl. The non-N-terminal lysine, which had an ε-NH_2 group that

TABLE IX. NON-N-TERMINAL LYSINE IN INSULIN

Sample NO.	Non-N-terminal lysine (g. lysine/16 g. nitrogen)		% Recovery
	Experiment	Reference[a]	
Modified FDNB method			
1	2.46	2.57	95.7
2	2.46	"	95.7
3	2.49	"	96.9
4	2.49	"	96.9
5	2.46	"	95.7
6	2.45	"	95.3
7	2.41	"	93.8
8	2.40	"	93.4
Mean	2.45	"	95.3
Modified TNBS method			
1	2.11	2.57	82.1
2	2.21	"	86.0
3	2.09	"	81.3
4	2.37	"	92.2
5	2.09	"	81.3
Mean	2.17	"	84.6

[a]Calculated from the structure given by Ryle, Sanger, Smith and Kital (45).

TABLE X. NON-N-TERMINAL LYSINE IN OVALBUMIN

Sample NO.	Non-N-terminal lysine (g. lysine/16 g. nitrogen)		% Recovery
	Experiment	Reference[a]	
Modified FDNB method			
1	5.71	6.60	86.5
2	6.30	"	95.5
3	6.30	"	95.5
Mean	6.10	"	92.5
Modified TNBS method			
1	4.83	6.60	73.2
2	4.97	"	75.3
3	5.19	"	78.6
4	5.22	"	79.1
5	5.02	"	76.1
6	5.05	"	76.5
Mean	5.05	"	76.5

[a]Calculated from the analysis given by Steven and Tristam (37).

TABLE XI. NON-N-TERMINAL LYSINE IN α-CHYMOTRYPSINOGEN A

Sample NO.	Non-N-terminal lysine (g. lysine/16 g. nitrogen)		% Recovery
	Experiment	Reference[a]	
Modified FDNB method			
1	6.12	7.59	80.6
2	5.67	"	74.7
3	6.00	"	79.1
4	6.01	"	79.2
5	5.90	"	77.7
6	5.47	"	72.1
7	5.39	"	71.0
Mean	5.79	"	76.3
Modified TNBS method			
1	4.40	7.59	58.0
2	4.99	"	65.7
3	4.71	"	62.1
4	4.71	"	62.1
5	4.95	"	65.2
6	4.95	"	65.2
Mean	4.79	"	63.1

[a]Calculated from the structure given by Brown and Hartley (46).

TABLE XII. NON-N-TERMINAL LYSINE IN LYSOZYME

Sample NO.	Non-N-terminal lysine (g. lysine/16 g. nitrogen)		% Recovery
	Experiment	Reference[a]	
Modified FDNB method			
1	3.64	4.35	83.7
2	3.61	"	83.0
3	3.64	"	83.7
4	3.40	"	78.2
5	3.87	"	89.0
6	3.60	"	82.8
7	3.55	"	81.6
Mean	3.62	"	83.2
Modified TNBS method			
1	3.90	4.35	89.7
2	3.77	"	86.7
3	4.06	"	93.3
4	4.32	"	99.3
5	4.32	"	99.3
6	4.12	"	94.7
Mean	4.08	"	93.8

[a]Calculated from the structure given by Canfield and Liu (47).

TABLE XIII. ANALYTICAL VALUES FOR WHEAT SAMPLES[a]

Percent	Wheat Sample NO.							
	6	7	9	10	11	12	15	16
1. Protein	14.0	12.6	15.0	15.4	14.8	12.8	14.1	14.2
2. Total lysine[b]	0.379	0.343	0.409	0.407	0.400	0.359	0.398	0.384
3. Available lysine[c]	0.354	0.325	0.339	0.326	0.356	0.302	0.361	0.352
4. Extracted protein	97.9	97.7	99.0	96.9	97.2	92.9	97.0	94.9
5. Available lysine corrected for % protein extraction(3/4)	0.362	0.333	0.342	0.336	0.366	0.325	0.372	0.371
6. Available lysine in total lysine (5/2)	95.5	97.1	83.6	82.6	91.5	90.5	93.5	96.6
7. Available lysine in wheat protein (5/1)	2.59	2.64	2.28	2.18	2.47	2.54	2.64	2.61

[a]All data based on 14% moisture basis and nitrogen-to-protein conversion factor of wheat based on 5.7.

[b]% Total lysine determined by Beckman Amino Acid Analyzer Model 120 C.

[c]Available lysine determined by modified FDNB method, the average value of two duplicates from each of two replicates.

TABLE XIV. ANALYTICAL VALUES FOR WHEAT SAMPLES[a]

Percent	Wheat Sample NO.							
	17	18	19	20	21	22	23	24
1. Protein	14.4	15.4	16.2	13.4	13.8	19.6	18.1	17.1
2. Total lysine[b]	0.419	0.436	0.453	0.370	0.375	0.489	0.456	0.436
3. Available lysine[c]	0.389	0.412	0.335	0.323	0.305	0.406	0.409	0.395
4. Extracted protein	96.0	96.4	101.1	98.9	98.7	99.8	98.9	99.0
5. Available lysine corrected for % protein extraction (3/4)	0.405	0.428	0.331	0.327	0.309	0.407	0.413	0.399
6. Avaliable lysine in total lysine (5/2)	96.7	98.2	73.1	88.4	82.4	83.2	90.7	91.5
7. Available lysine in wheat protein (5/1)	2.81	2.78	2.04	2.44	2.24	2.08	2.28	2.33

[a]All data based on 14% moisture basis and nitrogen-to-protein conversion factor of wheat based on 5.7.

[b]% Total lysine determined by Beckman Amino Acid Analyzer Model 120 C.

[c]Available lysine determined by modified FDNB method, the average value of two duplicates from each of two replicates.

reacted with FDNB, was determined by thin-layer chromatography. FDNB-lysine is the available lysine according to Carpenter's definition (4,5). Values in line 5 are available lysine in wheat corrected for protein extraction loss. The difference between the values in lines 2 and 5 is assumed to be unavailable lysine which is presumably "inactivated" through the formation of enzyme-resistant bonding involving the ε-NH_2 group. Inactivated or bound lysine, therefore, is unreactive to FDNB reagent. In the determination of available lysine by the FDNB reaction, it must be remembered that the N-terminal lysine moiety of the polypeptide is lost even though its ε-NH_2 group is free (and therefore "available"), because the terminal lysine forms ether-soluble di-DNP-lysine and is removed by the ether extraction. The proportion of such lysine to the total lysine is rather small, provided the starting sample is not highly degraded to amino acids or short chain peptides.

The mean per cent recovery of non-N-terminal lysine determined by the modified FDNB method was insulin 95.3%, ovalbumin 92.5%, and α-chymotrypsinogen A 76.3% (Tables IX, X and XI). The mean per cent recovery of non-N-terminal lysine determined by Matheson's second procedure (12) was insulin 91.6%, ovalbumin 85.8%, and α-chymotrypsinogen A 66.1%. The modified FDNB method gave better recovery than Matheson's method. This may be due to the better separation of ε-DNP-lysine by thin-layer chromatography than by column chromatography.

The modified FDNB method had other advantages over Matheson's FDNB method:

(a) The procedure of the thin-layer chromatography was simpler than that of column chromatography.

(b) Four to five samples in duplicates can be applied in a single

thin-layer plate while a duplicate sample requires two columns. Therefore, thin-layer chromatography was time saving.

(c) One operator can take care of many TLC plates at a time, but the columns require constant attention. This gives thin-layer chromatography another time-saving advantage.

Satisfactory results were obtained with the modified FDNB method used to determine the content of non-N-terminal lysine in purified protein.

Many workers (3,4) have reported that the size of the ground sample affected the reactivity of lysine with FDNB. Therefore, the direct use of ground samples in dinitrophenylation raises a question of whether such samples would react completely with FDNB.

The quantitative extraction of wheat protein by 0.2% NaOH and directly using the protein extract for dinitrophenylation has several advantages over the use of ground samples for dinitrophenylation:

(a) The efficiency of the dinitrophenylation was greater with wheat protein extract.

(b) The amount of carbohydrate was greatly decreased, the interference of the carbohydrate was proportionally reduced, thus better separation and higher recovery of ε-DNP-lysine was obtained.

The acid hydrolysis of the TNP-protein in the standard procedure (33) was not complete. Six wheat samples (NO. 19, 20, 21, 22, 23 and 24) analyzed by the TNBS procedure had two unknown yellow spots located above ε-TNP-lysine on the thin-layer chromatogram (Fig. 6). The two unknown yellow spots were neither α-TNP-arginine nor α-TNP-histidine, but may be the acid-soluble small TNP-peptides mentioned in the TNBS method. These two unknown yellow spots are larger than the ε-TNP-lysine yellow spot produced from wheat samples. Therefore, the measuring of acid-soluble TNP-amino

acids as the ε-TNP-lysine will overestimate the available lysine. The TNBS method also overestimated available lysine in some purified proteins (insulin and ovalbumin) and certain flour samples (fish flour, soy flour, chickpea flour, broad bean flour and powder sodium caseinate) (Fig. 5), because of the formation of the two unknown yellow spots (TNP-compounds) which could intensify the absorbance of ε-TNP-lysine. If other acid-soluble TNP-amino acids beside ε-TNP-lysine, such as α-mono-TNP-cysteic acid, α-TNP-arginine, and α-TNP-histidine, remain in the aqueous solution, they will have the same maximum absorption as ε-TNP-lysine at 346 nm. wavelength. Therefore, the TNBS method may overestimate the available lysine in protein foodstuffs.

The non-N-terminal lysine in four purified proteins obtained by modified FDNB method and modified TNBS method are compared in Tables IX, X, XI and XII. The FDNB method gave higher recovery than the TNBS method in the analysis of insulin, ovalbumin and α-chymotrypsinogen A. That may be due to the loss of ε-TNP-lysine in the form of an acid-soluble small TNP-peptide. With lysozyme, the FDNB method gave lower recovery than the TNBS method. The reason is not known.

It seems that FDNB is more reactive than TNBS, because the flouro group in FDNB is more active than the $-SO_3H$ group in TNBS for aromatic nucleophilic substitution (48). TNBS reacts with only primary amino groups of amino acids and peptides in an aqueous solution at pH 8 and at room temperature. No reaction occurs with the imidazole nitrogen of histidine, the quanidium group of arginine, or the hydroxyl group of tyrosine or threonine (28). At the room temperature, FDNB reacts with protein in the presence of ethanol at neutral to alkaline pH. The dinitrophenylation takes place at the free amino group, the imidazole group of histidine, the phenolic-hydroxyl group of tyrosine and the thiol group of cysteine (10).

Krull et al. (49) described the conformation of protein chains in wheat flour, showing that the protein backbone was not usually arranged in a linear fashion, but may be in a random, helical, or highly folded conformation. The protein chain may even fold into a compact ball because of electrostatic forces, hydrophobic attractions and hydrogen bonding between different side chains. The salt-soluble proteins in wheat were often highly folded. The molecular size of TNBS is larger than that of FDNB; therefore, the ε-amino group of lysine in wheat protein would be more available to FDNB. Further, the gliadin protein of wheat is soluble in 70% ethanol. The reaction conditions probably favor dinitrophenylation over trinitrophenylation of wheat proteins.

SUMMARY AND CONCLUSION

The determination of total nitrogen has been the most valuable tool for the estimation of nutritive value of protein during the past hundred years. Today, total nitrogen still is an important factor for estimating the nutritive value of food.

The nutritional value of proteins also depends on the amino acids that they yield on digestion. Since the requirement for each individual essential amino acid is fairly well known, it might seem that one only has to carry out a standard chemical analysis for the amino-acid composition of a diet, and its exact nutritional value, from the point of view of protein, could then be estimated. In practice it is often difficult to estimate the nutritional value of a protein based on its amino acid composition largely because a proportion of its amino acids such as lysine in the dietary constituents may be biologically unavailable. Because of this and of reports showing a correlation between values obtained with FDNB and the results of feeding tests, there has been widespread interest in the determination of FDNB-available lysine as a quality control procedure (50).

Matheson's FDNB method (12), and Kakade and Liener's TNBS method (33) have been widely used for the determination of available lysine content of proteins and protein foodstuffs. The procedure of Matheson's FDNB method consists of dinitrophenylation, acid hydrolysis, solvent partition, column chromatography, and colorimetry. The procedure of Kakade and Liener's TNBS method includes trinitrophenylation, acid hydrolysis, solvent partition, and colorimetry.

In the modified FDNB method, column chromatography was replaced by thin-layer chromatography to separate ε-DNP-lysine (available lysine) from

acid soluble DNP-amino acids in order to simplify the procedure and to save the operating time. In the modified TNBS method, thin-layer chromatography was included to obtain a complete separation of ε-TNP-lysine (available lysine) from acid soluble TNP-amino acids.

The procedures in which the separated substance is eluted from the adsorbent and quantitatively determined by a Spectrophotometer are in general use today. But the disadvantages in comparison with direct evaluation on the layer, are the greater time necessary and a certain unrecoverable amount of substance. Also, because the amount of the separated available lysine (ε-DNP-lysine or ε-TNP-lysine) on the TLC chromatograms was so small; the intensity of the yellow color of the available lysine solution eluting from the adsorbent with 1 N HCl was not strong enough for spectrophotometry. Therefore, it is advantageous to measure the available lysine directly on the TLC chromatograms by a Densitometer.

A comparative study was made on the determination of available lysine in four purified proteins (insulin, ovalbumin, α-chymotrypsinogen A and lysozyme) by the modified method and the original ones. The modified FDNB method gave a better separation of ε-DNP-lysine by thin-layer chromatography than by column chromatography. The modified TNBS method was faced incomplete acid hydrolysis problem as Kakade and Liener's TNBS method, resulting in lower recovery of ε-TNP-lysine. Therefore, the TNBS method was not used to determine the available lysine in wheat.

For the determination of available lysine in wheat, wheat protein extract instead of ground wheat sample was used for dinitrophenylation, in order to increase the efficiency of the dinitrophenylation, and to reduce the carbohydrate interference for better separation and higher recovery of ε-DNP-lysine. Sixteen wheat samples were examined for available lysine by the modified FDNB method.

Two grams of the hammer-milled finer wheat powder were extracted by 50 ml. of 0.2% NaOH. One ml. of the protein extract as each replicate was dinitrophenylated with 1-fluoro-2,4-dinitrobenzene (FDNB) in the presence of ethanol. The DNP-proteins were acid hydrolyzed, and the resulting DNP-amino acids were divided into ether or acid soluble DNP-amino acids by a solvent partition. The ε-DNP-lysine was separated from acid soluble DNP-amino acids by one-dimensional ascending thin-layer chromatography, using TLC plate coated with 0.5 mm. layer of silica gel G (20 by 20 cm.), and a solvent system of n-propanol-34% ammonium hydroxide (70:30, v./v.). The samples' acid soluble DNP-amino acids as well as standard ε-DNP-l-lysine HCl were spotted on the same plate. After a 15 cm. solvent migration (about 2 hr. 40 min.), the plate was removed and dried. Direct photometric determination of ε-DNP-lysine separated by TLC was conducted by use of Photovolt Densitometer Model 530, with scanning stage and varicord recorder. The peak area was measured using Gelman Planimeter. The amount of ε-DNP-lysine in wheat samples was then calculated according to the calculating formulas listed in page 27. The analytical data showed that the per cent available lysine to total lysine of the sixteen wheat samples was varied from 73.1% to 98.2%. This wide variation found in the present study, could serve an important notice that in selecting a high-lysine wheat variety one should pay attention not only to its total lysine but also to its available lysine content.

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DETERMINATION OF LYSINE IN WHEATS BY EXTRACTION, DINITROPHENYLATION AND THIN-LAYER CHROMATOGRAPHY

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Cereals are the major food for a great many people in the world. Wheat, rice and corn are the major cereals and all are limited in certain essential amino acids, especially lysine. Lysine is known to be the most limiting amino acid in the protein of wheat. Further, some of lysine in wheat and wheat products may become unavailable nutritionally. Therefore, it seems desirable, from a nutritional standpoint, to estimate the available lysine content in wheat and wheat products.

Matheson's FDNB method, and Kakade and Liener's TNBS method have been widely used for the determination of available lysine content of proteins and protein foodstuffs. The procedure of Matheson's FDNB method consists of dinitrophenylation, acid hydrolysis, solvent partition, column chromatography, and colorimetry. The procedure of Kakade and Liener's TNBS method includes trinitrophenylation, acid hydrolysis, solvent partition, and colorimetry.

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