CHEMICAL AND ENZYMIC ASSAYS
FOR AVAILABLE LYSINE

by

MARILUZ HOLGUIN
B.Sc., Universidad Javeriana, 1974

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
MASTER OF SCIENCE
in
The Faculty of Graduate Studies
(Department of Food Science)

We accept this thesis as conforming to
the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
FEBRUARY, 1979

© Mariluz Holguin, 1979
In presenting this thesis in partial fulfilment of the requirements for 
an advanced degree at the University of British Columbia, I agree that 
the Library shall make it freely available for reference and study. 
I further agree that permission for extensive copying of this thesis 
for scholarly purposes may be granted by the Head of my Department or 
by his representatives. It is understood that copying or publication 
of this thesis for financial gain shall not be allowed without my 
written permission.

Mariluz Holguin

Department of Food Science

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date February 2, 1979
ABSTRACT

Lysine is of prime nutritional significance since it is the first limiting amino acid in many foods of plant origin and is easily rendered unavailable upon heat processing or upon unfavourable storage conditions. The term "available lysine" refers to forms of lysine which contain free ε-amino groups within the peptide chain. Once the ε-amino group is blocked, lysine becomes unavailable since it cannot be hydrolyzed by proteolytic enzymes.

The availability of lysines in casein, lysozyme, β-lactoglobulin, acid solubilized gluten and whole egg was determined by the pepsin pancreatin digestion test, the dinitrobenzene sulfonic acid (DNBS) and the trinitrobenzene sulfonic acid (TNBS) methods. The results were compared to the fluorodinitrobenzene (FDNB) method. Good agreement was obtained between the FDNB official method and the DNBS technique, with a correlation coefficient of 0.989. When the TNBS method was compared to the FDNB difference technique, a correlation coefficient of 0.988 was found.

The pepsin pancreatin digestion test indicated the relative amount of lysine released by the enzymes under the conditions specified by the test. A correlation coefficient of 0.995 was found between the FDNB official method
and the enzymatic test.

The specificity of DNBS for the \( \epsilon \)-amino group of lysine was determined using \( \alpha \)- and \( \epsilon \)-formyl-lysines, L-lysine, L-lysyllysine, L-lysylalanine and ribonuclease-S-peptide. DNBS was found to react mainly with \( \epsilon \)-amino group but with a slight reactivity with \( \alpha \)-amino group. However, in the case of proteins with several \( \epsilon \)-amino groups and N-terminal lysine, the contribution of the \( \alpha \)-amino group to the results becomes negligible.

The DNBS method was found to be the simplest and most reliable method for determination of available lysine, for the following reasons: a) it does not require acid hydrolysis of the proteins; b) a large number of samples can be analyzed simultaneously in a few hours, and c) it does not require expensive and lengthy chromatographic amino acid analysis.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>i.</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii.</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv.</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vi.</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii.</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE SURVEY ON METHODS FOR DETERMINATION OF LYSINE AVAILABILITY.</td>
<td>8</td>
</tr>
<tr>
<td>A. Bioassays</td>
<td>8</td>
</tr>
<tr>
<td>B. Microbiological Assays</td>
<td>12</td>
</tr>
<tr>
<td>C. Chemical Methods</td>
<td>16</td>
</tr>
<tr>
<td>1. The fluorodinitrobenzene method (FDNB)</td>
<td>17</td>
</tr>
<tr>
<td>2. The trinitrobenzene sulfonic acid method (TNBS)</td>
<td>28</td>
</tr>
<tr>
<td>3. Guanidination</td>
<td>35</td>
</tr>
<tr>
<td>4. Acrylonitrile</td>
<td>36</td>
</tr>
<tr>
<td>5. Methyl acrylate</td>
<td>36</td>
</tr>
<tr>
<td>6. Ethylviny sulfone</td>
<td>37</td>
</tr>
<tr>
<td>7. 2-Chloro-3,5-dinitropyridine</td>
<td>37</td>
</tr>
<tr>
<td>8. Sodium borohydride</td>
<td>38</td>
</tr>
<tr>
<td>9. $^{19}$F NMR</td>
<td>39</td>
</tr>
<tr>
<td>10. The sodium dinitrobenzene sulfonate method (DNBS)</td>
<td>42</td>
</tr>
<tr>
<td>11. Fluorescamine</td>
<td>48</td>
</tr>
<tr>
<td>12. Dye-binding</td>
<td>48</td>
</tr>
<tr>
<td>13. Chemical methods in the determination of available lysine in materials that have undergone Maillard reactions</td>
<td>51</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (Continued)

LITERATURE SURVEY ON METHODS FOR DETERMINATION OF LYSINE AVAILABILITY (Continued)

D. Enzymatic Methods 54

MATERIALS AND METHODS 65

A. Materials 65
B. Nitrogen Determination 66
C. Amino Acid Analysis 66
D. Lysine Determination by the Dinitrobenzene Sulfonic Acid Method 67
E. Lysine Determination by the Trinitrobenzene Sulfonic Acid Method 74
F. Lysine Determination by the Dinitrofluorobenzene Method 79
G. Enzymatic Digestion Test 85

RESULTS AND DISCUSSION 89

A. The Dinitrobenzene Sulfonic Acid Method 89
B. The Trinitrobenzene Sulfonic Acid Method 96
C. The Fluorodinitrobenzene Method 101
D. The Pepsin Pancreatin Digestion Test 105

GENERAL DISCUSSION 110

CONCLUSION 120

REFERENCES CITED 122

APPENDIX 146
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Description</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(a)</td>
<td>Structure of free lysine. From: Bodwell, 1976.</td>
<td>2</td>
</tr>
<tr>
<td>1(b)</td>
<td>Lysine as it would exist in a peptide chain. From: Bodwell, 1976.</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Reaction of FDNB with an amino acid. From: Hall et al., 1974.</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td>Reaction of TNBS with an amino acid. From: Hall et al., 1973.</td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>$^{19}$F NMR spectrum of the reaction products of a protein and S-ethyl trifluorothioacetate in dimethyl sulfoxide solution.</td>
<td>41</td>
</tr>
<tr>
<td>5</td>
<td>Structure of the reactive dye remazol brilliant blue R. From: Ney and Wirotama, 1970.</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>The dinitrobenzene sulfonic acid method for determination of available lysine.</td>
<td>72</td>
</tr>
<tr>
<td>7</td>
<td>Standard curve for available lysine determination for a sample treated with dinitrobenzene sulfonic acid.</td>
<td>73</td>
</tr>
<tr>
<td>8</td>
<td>The trinitrobenzene sulfonic acid method for the determination of available lysine.</td>
<td>77</td>
</tr>
<tr>
<td>9</td>
<td>Standard curve for available lysine determination for a sample treated with 2,4,6-trinitrobenzene sulfonic acid</td>
<td>78</td>
</tr>
<tr>
<td>10</td>
<td>Refluxing system used in the preparation of protein hydrolysates</td>
<td>82</td>
</tr>
<tr>
<td>11</td>
<td>The dinitrofluorobenzene method for determination of available lysine (difference technique). From: Blom et al., 1967.</td>
<td>84</td>
</tr>
<tr>
<td>Figure No.</td>
<td>Description</td>
<td>Page No.</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>12</td>
<td>Comparison between the FDNB difference technique and the DNBS method. The % of lysine is determined by g lysine/100 g protein.</td>
<td>112</td>
</tr>
<tr>
<td>13</td>
<td>Comparison between the FDNB difference technique and the TNBS method. The % of lysine is determined by g lysine/100 g protein.</td>
<td>113</td>
</tr>
<tr>
<td>14</td>
<td>Comparison between the FDNB difference technique and the enzymatic digestion test. The % of lysine is determined by g lysine/100 g protein.</td>
<td>114</td>
</tr>
<tr>
<td>15</td>
<td>Comparison between the enzymatic digestion test and the DNBS method. The % of lysine is determined by g lysine/100 g protein.</td>
<td>115</td>
</tr>
<tr>
<td>16</td>
<td>Comparison between the enzymatic digestion test and the TNBS method. The % of lysine is determined by g lysine/100 g protein.</td>
<td>116</td>
</tr>
<tr>
<td>17</td>
<td>Comparison between the TNBS and the DNBS methods. The % of lysine/100 g protein.</td>
<td>117</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table No.</th>
<th>Description</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reagents used and derivates formed in Direct Methods.</td>
<td>18</td>
</tr>
<tr>
<td>2</td>
<td>Reagents used and derivates formed in Indirect Methods.</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>Protein content of samples assayed for lysine availability.</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>Available lysine content of protein samples as determined by the dinitro-</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>benzene sulfonic acid (DNBS) method.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Reaction of amino acids and peptides with dinitrobenzene sulfonic acid</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>(DNBS).</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Available lysine content of protein samples as determined by the trinitro-</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>benzene sulfonic acid (TNBS) method.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Available lysine content of protein samples as determined by the fluoro-</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>dinitrobenzene (FDNB) method.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Amount of lysine released from protein samples subjected to pepsin-pancreatin</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>digestion.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Lysine availability determined by the FDNB official procedure, the DNBS and</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>the TNBS methods, and the enzymatic test.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Standard deviation of the random error (in g lysine/100 g protein) in the</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>available lysine methods, as estimated with Deming's procedure.</td>
<td></td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. S. Nakai, Chairman of my committee for his supervision and encouragement in the preparation of this thesis. Also to the other members of my committee: Drs. Powrie, Vanderstoep and Beames my sincere thanks.

To Valerie (technician) I am truly grateful for her co-operation and assistance.

I should also like to thank my husband for his help and encouragement in the preparing of this thesis.
INTRODUCTION

The nutritional quality of a food protein is dependent not only on the amino acid composition and digestibility of the protein itself but also upon the physiological availability. Availability can be defined as the amount or percentage of a given amino acid in the food which is utilized for protein synthesis in the organism (growth and maintenance) when the said amino acid is the only limiting factor of the diet. Growth may therefore be considered to be a suitable index of availability.

Dietary lysine insufficiency often limits growth. In many foods, lysine is limiting not only because relatively small amounts are incorporated in the proteins during biosynthesis but also because of secondary chemical changes due to factors such as light, heat, alkali, and reducing sugars, as a result of which lysine becomes nutritionally unavailable.

Lysine in its free form has a free $\alpha$-amino group, a free $\epsilon$-amino group and a free carboxyl group. In most proteins, 95 to 100% of the lysine is linked to other amino acids through peptide linkages involving the $\alpha$-amino and carboxyl groups (Figure 1). Only the $\epsilon$-amino group is free to react with other compounds such as sugars or amide groups of other amino acids.
FIGURE 1. (a) Structure of free lysine. From: Bodwell, 1976.

\[
\begin{align*}
\text{\(\varepsilon\)-amino group} & \rightarrow \text{NH}_2 \\
& \quad \text{CH}_2 \\
& \quad \text{CH}_2 \\
& \quad \text{CH}_2 \\
& \quad \text{CH}_2 \\
& \quad \text{H}_2\text{N} - \text{C} - \text{COOH} \\
& \quad \text{H} \\
\text{\(\alpha\)-amino group}
\end{align*}
\]
FIGURE 1. (b) Lysine as it would exist in a portion of a peptide chain. From: Bodwell, 1976.

Threonine  Lysine  Glycine  Methionine
Lysine is nutritionally available if its $\epsilon$-amino group is free. If the $\epsilon$-amino group is blocked through a chemical bond, the segment of the protein near the affected lysine residue may not be digested.

If the protein is broken down to amino acids or dipeptides, the reacted lysine may not be absorbed from the gastrointestinal tract. Even if absorbed, the lysine having a 'derivatized' $\epsilon$-amino group may not be utilized but rather excreted in the urine.

Lysine may also be buried in a protein matrix of a particular sequence or conformation which is slow to hydrolyze or is not hydrolyzed at all by animal proteases. Such lysine may or may not appear as chemically available by chemical methods and yet be totally unavailable nutritionally.

Proteolysis within the intestinal lumen is largely carried out by the pancreatic proteolytic enzymes. Trypsin and chymotrypsin account for 78% of the total proteolytic activity. Trypsin acts on a protein at either an arginine residue or a lysine residue which has a free amino group. The action of other proteases is only partially inhibited when the $\epsilon$-amino group of lysine is blocked.

Under severe or prolonged heating conditions, the $\epsilon$-amino group of lysine can react with the $\gamma$-carboxyl group of glutamic acid or the $\beta$-carboxyl group of aspartic acid on another protein or within the same protein molecule.
As a result, a so called isopeptide bond is formed, which is resistant to hydrolysis by gut proteases. Proteolytic enzymes are believed to be specific for the $\delta,\lambda$-peptide linkages. The aspartyl- or glutamyl-lysine remains after proteolysis. However, Mauron (1972) and Carpenter and Booth (1973) have shown that these derivatives can be utilized as sources of lysine by the rat. The dipeptides are probably absorbed as such through the intestinal wall and hydrolyzed in the kidneys. The presence of numerous such cross-linkages might be expected to at least reduce the rate of protein digestion, possibly by steric hindrance of the sites of attack by digestive enzymes.

Another reaction of lysine is cross-linkage with dehydroalanine residues. Dehydroalanine is formed by heating serine or cysteine under alkaline conditions. The dehydroalanine thus formed reacts with the $\epsilon$-amino group to form lysinoalanine (LAL). It appears that lysinoalanine is not nutritionally available since 38-65% of the LAL ingested by rats in the form of alkali-treated soybean casein or soybean meal was recovered in the feces (deGroot and Slump, 1969).

A more common reason for losses of available lysine may be reaction with carbohydrates. Both reducing and non-reducing sugars react with lysine. El-Nockrashy and Frampton (1967) showed that sucrose, raffinose and trehalose all react with lysine to render it unavailable. Maillard
reaction in which the ε-amino group of lysine reacts with aldehyde groups of reducing sugars, or with carbonyl groups of lipids, is very well known to decrease lysine availability.

Thompson et al. (1976) have studied the loss rate of available lysine during thermal processing of a soy model system. Available lysine loss went through three phases. The first phase was characterized by a rapid loss in which 30 to 60% of the available lysine was made unavailable. Phase two showed a statistically significant (98% confidence) increase in available lysine when measured by Carpenter's fluorodinitrobenzene procedure (FDNB). The third phase was characterized by a stabilization of FDNB available lysine. The reaction resulting in the initial rapid phase occurred according to first order reaction kinetics. Wolf and Thompson (1977) utilized a model system consisting of protein, glucose, and microcrystalline cellulose. It was found that glucose and temperature had the dominant effect on predicting the specific reaction rate ($k_T$). Water activity and pH also influenced the prediction of $k_T$ but they acted though an interaction with glucose. Jokinen et al. (1976) developed an equation which predicts lysine availability as a function of pH, water activity, glucose, starch, and sucrose in phase three. Thermal loss of available lysine in fortified rice meal was kinetically assessed by
Tsao et al. (1978).

Because of the extreme importance of lysine in the diet, and the additional considerations of protein damage and internal cross-linkages following some types of processing, several methods have been proposed for estimating nutritionally available lysine. These methods can be categorized in four groups: biological evaluation studies, in vitro digestion methods, and microbiological and chemical analyses. Much effort has been made to establish reliable chemical methods which are quick and suitable for routine assay.

The object of this study was to determine lysine availability with two of the most popular chemical methods, the dinitrobenzene sulfonic acid (DNBS) and the trinitrobenzene sulfonic acid (TNBS) techniques, and to compare the results with the fluoro dinitrobenzene (FDNB) AOAC official method.

Enzymatic digestion assays were done in an attempt to correlate chemical and biological availabilities. For example, it has been demonstrated that, in some cases, chemical determinations do not show the extent of protein digestibility or the presence of trypsin inhibitors.
METHODS FOR DETERMINATION OF LYSINE AVAILABILITY

A. BIOASSAYS

The ultimate standards for the in vitro methods of measuring amino acid availability are bioassays with animals. Animal assays are based on the observation that the amount and quality of a protein can result in a gain or loss of body substance. Such gain or loss can be identified by a change in body weight or by a change in a body component which often is nitrogen content.

The Protein Efficiency Ratio (PER), which is the official AOAC procedure, and the Net Protein Ratio (NPR) are based on weight changes. The methods based on body nitrogen change are the Net Protein Utilization (NPU) where carcass nitrogen is measured, and the Biological Value (BV) where nitrogen balance is measured.

PER is the slope of the line relating weight gain of a rat to protein intake consumed under standardized experimental conditions. Despite its long history, wide usage, and official status, PER is not a good assay procedure (Hegsted and Chang, 1965; Hegsted and Samonds, 1978). Of the criteria defined for a valid bioassay, i.e., precision, reproducibility, statistical validity, proportionality, simplicity and low cost (Hegsted and Samonds, 1978) PER could be considered as meeting only the criterion of simplicity. Some improvement can be made by inclusion of a
group of animals consuming a non-protein diet for a similar period. When modified in this way the procedure is called Net Protein Ratio (NPR) (Bender and Doell, 1957). A modification of NPR called Relative NPR (RelNPR) is recommended by the recent National Academy of Sciences - National Research Council Committee on Protein Evaluation (Young and Pellett, 1978) as a useful procedure if a multiple-point assay cannot be performed. Relative NPR is performed in an identical manner as NPR, but the results are expressed relative to the value obtained with an 8% lactalbumin diet taken as 1.00 or 100.

Bioassays are classed as either two-point or multiple-point assays. With two point assays, a straight line connecting the two points must be assumed. Studies with both rats (Hegsted and Neff, 1970; Hegsted, 1971) and humans (Young et al., 1977) have shown this assumption not to be entirely valid, especially for poor quality proteins. Multiple-point procedures involve measurements of the response obtained (weight, body water, or body nitrogen) in relation to protein consumed at several levels of protein consumption. These levels must be selected with care, since only for a small range can a straight line be assumed; at high levels more and more protein would be deaminated and used for energy, while at very low levels deviation from linearity could occur because of such phenomena as adaptive responses, with
consequently more efficient utilization of amino acids. This is essentially true when the limiting amino acid is lysine.

Very good correlations are found between bioassays and amino acid score for proteins having a biological value greater than 40 (Pellett, 1978). The relationship varies with the limiting amino acid below this level (Bender, 1961; Hegsted, 1971). Proteins completely lacking in lysine can still have a BV equal to 40, and proteins lacking other amino acid can have values significantly above zero. Young and Scrimshaw (1977) have shown that all animals, including man, have the ability to reutilize amino acids for the synthesis of proteins. For the human about 5-6 g of protein are synthesized for each g of protein allowance. Not all the amino acids behave in the same way; an absence of valine or total sulfur amino acids gives the expected zero response for BV, with the other essential amino acids behaving in an intermediate manner. While bioassay procedures such as the Relative Protein Value (RPV) (Young and Pellett, 1978), can give more realistic quality estimates for poor quality proteins, no bioassay is exempt from this problem. An animal can conserve and recycle lysine for a limited period by slowing down lysine oxidation (Brooks et al., 1972). Fortunately, in practice very few proteins or real dietaries have these very low levels of essential amino acids.
The content of available lysine can be measured by comparing the performance of animals receiving a basal diet supplemented with the test material or with pure lysine. The basal protein source has been maize with cystine and tryptophan (Wilder and Kraybill, 1947), wheat (Howard et al., 1958; Morrison et al., 1963; Mauron and Mottu, 1962), wheat gluten (Pion and Rérat, 1962) or a mixture of wheat and barley (Brüggemann et al., 1969). It is essential that lysine should be a limiting factor in the diets of test animals, otherwise they will not be sensitive to changes in the level of available lysine.

Although a basal diet may be specifically deficient in lysine (in the sense that this is the only amino acid that will elicit a growth response when added to the diet), the magnitude of the response to a test supplement can also be affected by the amounts of other amino acids that it contains, through changing the amino acid balance of the diet as a whole (Muelenaere et al., 1967).

Discrepancies have been found consistently according to the dose response relations used for calculation of results which have been: A. % lysine in the diet: weight gain/day; B. % lysine in the diet: weight gain/g food eaten, and C. g lysine eaten: weight gain/day. In general A has resulted in lower calculated values (Calhoun et al., 1960; Carpenter et al., 1963; Guo et al., 1971). B and C have
been preferred because they give values less affected by amino acid balance (Netke et al., 1969) and by other compounds influencing food consumption (Gupta et al., 1958).

Carpenter et al. (1963) and Smith and Scott (1965) obtained values with chicks for the potency of undamaged fish protein that were higher than the corresponding total lysine values obtained by chemical analysis. With well processed soya meal, Combs et al. (1968) found nearly 100% availability, whereas Netke and Scott (1970) obtained results equivalent to only 74 to 80% of the total values. Thus, it is not possible to make reliable comparisons between materials when the potencies are assessed in different laboratories and, even for within-experiment comparisons there is a need for caution and replication. Unfortunately, growth assays remain the only direct means to check the validity of claims for the nutritional relevance of values obtained by other procedures.

B. MICROBIOLOGICAL ASSAYS

A great advance in the determination of available amino acids was achieved by the introduction by Ford (1960) of microbiological methods. Microbiological assays are rapid, comparatively cheap and provide a means of testing
large number of samples in a short time. They can be used for three main purposes: 1) determination of amino acid availability after enzyme hydrolysis; 2) determination of total amino acids after acid hydrolysis; and 3) determination of protein quality, expressed as Relative Nutritive Value (RNV), as assessed by the growth of the test organism relative to that with a standard protein, usually casein or whole eggs (Bender, 1969; Ford, 1965; Szklarska-Cyganska and Kakowska-Lipinska, 1970). RNV is a relative measure of the Tetrahymena bioassay and not meant to relate to the rat RNV.

The microorganisms are cultivated on a medium containing all amino acids except the one to be detected in an unknown sample. If a strain is used for which this amino acids is indispensable, the cells will not multiply. The growth of the culture is measured by the turbidity produced by the growing microorganisms, or by determination of the lactic acid formed. If the extent of growth, or the amount of lactic acid is plotted against the amount of amino acid added, a standard curve is obtained which makes it possible to determine the amino acid content of the unknown sample by graphic interpolation.

The test microorganisms used show a pattern of requirement for exogenous amino acid, broadly similar to that of higher animals.
Ford (1962) has demonstrated the usefulness of Streptococcus zymogenes as an assay organism for most of the essential amino acids, since it has considerable proteolytic powers and, when test materials are given a preliminary partial digestion with papain, the values obtained for available methionine show a general similarity to values from animal assays (Miller et al., 1965). Unfortunately this organism does not require lysine.

Determination of protein quality using the growth of Tetrahymena pyriformis as an index was demonstrated by Rockland and Dunn (1949). Tetrahymena unlike S. zymogenes requires lysine but it also requires serine which is not an indispensable amino acid for higher animals. Viswanatha and Liener (1955) showed a need for predigestion of the protein sample prior to its addition to Tetrahymena culture medium. They showed that the proteinases present in Tetrahymena were unable to attack native globular proteins and that Tetrahymena was not affected by trypsin inhibitor or hemaglutinin components of raw soybean.

Fernell and Rosen (1956) also studied the Tetrahymena bioassay as a method for determining protein quality. Organism count and ammonia production over a four-day incubation were used as a means of obtaining a Relative Nutritive Value (RNV). No predigestion of the samples was employed, but the proteins were defatted because of
the possible growth inhibition from certain fatty acids (Rolle, 1973).

Landers (1975) who used a 3-hr pepsin predigestion and Frank et al. (1975) who used a 24-hr trypsin-bromelain predigestion of the food samples prior to their incorporation into the Tetrahymena growth media, reported high correlation between the Tetrahymena growth and the PER of the food protein.

Tetrahymena grows poorly in media containing free amino acids. A solution of amino acids simulating the composition of egg protein has an RNV of 50 to 60 as against 100 for the intact protein.

The microorganism does not produce acid, and the response has usually been measured by laborious cell counts. Although the protozoa are too heavy to remain in suspension for an ordinary turbidity reading, Shorrock (1972) found that with controlled autoclaving the organisms break up in a characteristic way that allows turbidity to be used as a measure of response.

Szmelcman and Guggenheim (1967) have pointed out that the distinction between available and unavailable amino acids by microbiological assay, is arbitrary and relates to the particular conditions of the assay.

Much more research concerning the applicability of Tetrahymena bioassay to a wide range of foods and its sensitivity to other non-protein food ingredients is needed before
the microorganism can be used as an effective tool to estimate food protein quality.

C. CHEMICAL METHODS

Since the first application of Sanger's technique (1945) to food by Carpenter and coworkers (1955a, 1957), all chemical methods for estimating the available lysine in a protein have been simple methods intended to estimate, either directly or indirectly, how much of the lysine is present which has a free ε-amino group.

The general approach for chemically estimating nutritionally available lysine can be summarized as follows:

a) A chemical agent is reacted with a protein and an acid stable derivative is formed between free ε-amino groups in the protein and the chemical agent.

b) The 'derivatized' protein is hydrolyzed to yield derivatized lysine plus other free amino acids (including non-derivatized, unavailable lysine).

c) The amount of derivatized lysine is determined (direct methods) and/or the amount of unreacted lysine is determined (indirect methods).

The direct methods currently in use or proposed are listed in Table 1, together with the reagents used and the derivatives formed. The indirect methods are listed in Table 2.

In high carbohydrate foods, the direct methods
(not considering methylisourea, DNBS and $^{19}\text{F NMR}$ methods) fail to yield satisfactory analytical results. Use of the indirect methods appear to circumvent these analytical problems. However, as reported by Bodwell (1976), the relationship of available lysine levels determined by chemical methods and the bioassays estimates, is not always satisfactory. For example, in wheat proteins, 85-95% availability for lysine, obtained by using chemical methods, is probably an overestimate for both rats and humans. The chemical estimates of available lysine may be overestimated because:

a) In the direct methods, the side reactions could increase the colorimetrically determined estimates of available lysine.

b) In both direct and indirect methods, some of the nutritionally available lysine may react with the chemical agents used to form acid stable derivatives and, if so, estimates of the available lysine level would be exaggerated.

In the following pages, the chemical methods listed in Tables 1 and 2 will be described. When possible, advantages and disadvantages for each one will be given.

1. The fluorodinitrobenzene method (FDNB).

The first good chemical method for the estimation of nutritionally available lysine was developed by Carpenter and his associates in 1955. The procedure was based on the work of Sanger who used the reagent 1-fluoro-2,
<table>
<thead>
<tr>
<th>Method</th>
<th>Reagent Used</th>
<th>Derivative Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDNB or Carpenter</td>
<td>FDNB (l-fluoro-2,4-dinitrobenzene)</td>
<td>DNP-lysine (€,N-dinitrophenyl-lysine)</td>
</tr>
<tr>
<td>TNBS</td>
<td>TNBS (2,4,6-trinitrobenzene sulfonic acid)</td>
<td>TNP-lysine (€,N-trinitrophenyl-lysine)</td>
</tr>
<tr>
<td>Guanidination</td>
<td>O-Methylisourea</td>
<td>Homoarginine</td>
</tr>
<tr>
<td>19F NMR</td>
<td>S-Ethyl-trifluorothioacetate</td>
<td>€,N-trifluoroacetyl-lysine</td>
</tr>
<tr>
<td>Available lysine</td>
<td>Acrylonitrile</td>
<td>€,N-cyanoethyl-lysine</td>
</tr>
<tr>
<td>Available lysine</td>
<td>2-Chloro-3,5-dinitropyridine</td>
<td>€,N-dinitropyridyl-lysine</td>
</tr>
<tr>
<td>Available lysine</td>
<td>Sodium borohydride</td>
<td>€-N,N-dimethyl-lysine</td>
</tr>
<tr>
<td>DNBS</td>
<td>DNBS (Sodium dinitrobenzene sulfonate)</td>
<td>DNP-lysine</td>
</tr>
<tr>
<td>Available lysine</td>
<td>Fluorescamine (4-phenylspiro-[furan-2-(3H)-1'-phthalan]-3,3'-dione)</td>
<td>Fluorescamine complex</td>
</tr>
<tr>
<td>Dye-binding</td>
<td>Remazol brilliant blue R</td>
<td>Dyed protein</td>
</tr>
<tr>
<td>Method</td>
<td>Reagent Used</td>
<td>Derivative Formed</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----------------</td>
<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Difference or Silcock</td>
<td>FDNB</td>
<td>DNP-lysine</td>
</tr>
<tr>
<td>Total Lysine Minus</td>
<td>TNBS</td>
<td>TNP-lysine</td>
</tr>
<tr>
<td>Unavailable Lysine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Lysine Minus</td>
<td>Methyl acrylate</td>
<td>$\epsilon,\epsilon,N,N$-dicarboxyethyl-lysine and $\epsilon,N$-monocarboxyethyl-lysine</td>
</tr>
<tr>
<td>Unavailable Lysine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Lysine Minus</td>
<td>Ethyl vinyl sulfone</td>
<td>$\epsilon,N$-ethylsulfonylethyl-lysine, or $\epsilon,\epsilon,N,N$-bisethylysulfonyl-ethyl-lysine</td>
</tr>
</tbody>
</table>
4-dinitrobenzene to determine the identities and number of the different N-terminal amino groups in proteins and peptides. Each free amino group became labelled, with a dinitrophenyl (DNP) group that resisted subsequent digestion with acid, so that each N-terminal amino acid unit that had originally reacted was converted to an $\alpha$-DNP-amino acid. These yellow compounds were separated and identified by column chromatography. In addition N-terminal lysine gave $\alpha,\epsilon$-di-DNP-lysine, and where lysine was present in other positions Sanger obtained $\epsilon$-DNP-lysine (abbreviated as DNP-lysine) which could also be separated by chromatography.

The reaction of FDNB with an amino acid is illustrated in Figure 2.

In Carpenter's original method the protein is dinitrophenylated and then hydrolyzed. A portion of the hydrolysate is extracted with ether and the extinction of the aqueous phase is read directly. Another portion is treated with methoxycarbonyl chloride to convert DNP-lysine to an ether-soluble derivative which is then extracted. The extinction of the remaining aqueous phase is read and the difference between the direct reading and the reading on the treated sample is taken as measuring $\epsilon$-DNP-lysine. A disadvantage of the method is that it estimates $\epsilon$-DNP-hydroxylysine, $\alpha$-DNP-ornithine and $\alpha$-DNP-arginine, as lysine. On the other hand, the method does not estimate N-terminal lysine or free lysine.
FIGURE 2. Reaction of 1-fluoro-2,4-dinitrobenzene (FDNB) with an amino acid. (From Hall et al., 1973).

\[
\text{R} \text{NH}_2-\text{CH-COOH} + \text{NaHCO}_3 + \text{HCl} \xrightarrow{\text{hydrolysis}} \text{Dinitrophenylated amino acid}
\]

\[
\text{I-Fluoro-2,4-dinitrobenzene}
\]
Carpenter's method is subject to interference by carbohydrate, especially in vegetable protein concentrates. Interference results in low and variable recoveries of ɛ-DNP-lysine. The effect is due to the destruction of DNP-lysine by carbohydrates during acid hydrolysis.

The procedure described by Carpenter and Ellinger in 1955 was modified by Carpenter (1960) to remove certain interfering compounds when applied to different types of feedstuffs.

The major criticism of Carpenter's FDNB is that a correction factor is required for samples that contain appreciable amount of carbohydrates, and it becomes difficult to apply with increasing concentrations of carbohydrates. The correction factor has been assessed by adding ɛ-DNP-lysine to duplicate samples at the beginning of the hydrolysis stage and taking it through the rest of the procedure. With animal materials the recovery was found to be 92%. Accordingly, all values determined should be multiplied by a factor of 100/92 = 1.09 to correct for losses during digestion. For vegetable foods the recovery of added ɛ-DNP-lysine was 60-85%. The use of the correction factor depends on the assumption that the ɛ-DNP-lysine that arises from the hydrolysis of protein is destroyed to the same extent as the added ɛ-DNP-lysine. Booth (1971) found that this assumption is invalid; ɛ-DNP-lysine in a protein is destroyed to a lesser extent than ɛ-DNP-lysine that has been
added as a free compound. Thus, the correction factor in the Carpenter method is less than formerly supposed. For materials that contain no carbohydrates a factor of 1.05 is more suitable than Carpenter's original 1.09, although very soluble albumins need the latter. For wheat and some other cereals the results need to be corrected by multiplying by 1.2. Between these two groups, lie beans, ground nut, and maize, requiring a factor of approximately 100/88 = 1.14.

The presence of carbohydrates results in very low recoveries of DNP-lysine, due to reduction of nitro groups in the dinitrophenylated amino acid to amino groups, which give derivatives without the characteristics used to measure DNP-lysine. Handwerck et al. (1960) reported that sugars are less destructive if they have previously been in contact with FDNB. Starch can be equally destructive and may not be inactivated by previous contact with FDNB (Erbersdobler and Zucker, 1964; El-Nockrashy, 1965). Destruction due to carbohydrates is lessened when the refluxing is carried out in the presence of another aromatic nitro compounds such as dinitrophenol, picric acid or FDNB itself (Blom et al., 1967; Matheson, 1968; Ruderus and Klöberg, 1970; Booth, 1971), presumably by competition with the nitro groups from DNP-lysine. This protection may be achieved by adding these compounds to the suspension before refluxing begins or by leaving the dinitrophenol and FDNB from the first stage of
the reaction in the reaction flask (Carpenter and Ellinger, 1955a; Carpenter, 1960). Matheson (1968) has shown that the quantities left in this way are sufficient to give the protective effect. With sugars carried through this procedure the destruction of DNP-lysine added immediately before hydrolysis is no more than 5-10% but it can be larger with starch (Matheson, 1968) or with structural carbohydrates (Booth, 1971).

Because of the technical difficulties encountered in the complete recovery of $\varepsilon$-DNP-lysine, an alternative approach has been to measure available lysine indirectly. The difference between the total lysine content of the sample measured after direct acid hydrolysis, and the residual lysine content measured after acid hydrolysis of the sample after blockage of the free $\varepsilon$-amino groups by reaction with DNFB, is assumed to be the available lysine of the sample. The main advantages of this technique, also known as the Difference technique or Silcock method, are that partial destruction of the derivative during acid hydrolysis does not cause difficulties, and that lysine itself is extremely stable to destruction by acid. However, automatic ion-exchange chromatography equipment is necessary for ease in estimation of lysine concentration.

Rao et al. (1963) first used the Difference technique as a check on the procedure they developed for determination
of available lysine in oilseed meal proteins. Roach et al. (1967) were the first to use the Difference procedure as a working replacement for the Carpenter FDNB method. They compared the methods of Carpenter (1960) and Rao et al. (1963) with the Silcock method for the estimation of available lysine in fishmeal and groundnut meal, and showed that the Silcock method is not subject to interference by carbohydrates. Compared to Carpenter's procedure, the Silcock method gave lower values for available lysine in groundnut meal. Similar results were reported by Ostrowski et al. (1970) relating to soybean meal.

Carpenter and co-workers (1957) directly measured the absorbance of the yellow ether-extracted acid hydrolisates of dinitrophenylated proteins in their estimation of available lysine. In a later modification (1960) Carpenter reduced some of the error in the analysis by reacting the 6-dinitrophenylhydrazone-lysine with methyl chloroformate to produce an ether soluble lysine derivative. The difference in color intensity of the hydrolysate before reaction with methyl-chloroformate and after reaction and extraction with ether was taken as a measure of available lysine.

Dinitrophenol is the major by-product of the dinitrophenylation of proteins. Conkerton and Frampton (1959), by taking advantage of the difference in absorbance of dinitrophenol at 360 nm in acid and alkaline media, were able to
correct for the quantity of dinitrophenol present in the protein hydrolysate. Other yellow substances might occur in the hydrolysate of some dinitrophenylated proteins (Handwerck et al., 1960) and introduce errors in a colorimetric estimation of available lysine. In addition, the brown humin pigments that invariably occur in acid hydrolysates may also be expected to contribute to the error. Many of the sources of error found in the earlier methods, are eliminated in the procedure described by Rao et al (1963) where ε-DNP-lysine is separated from dinitrophenol and other yellow derivatives of the reaction between DNFB and the meal proteins, as well as from the brown humin products of acid hydrolysis, through the use of an ion-exchange column that is developed with a mixture of methyl ethyl ketone and aqueous HCl.

Blom et al. (1967) also made use of a chromatographic separation technique to purify the resulting ε-DNP-lysine. The acid hydrolyzed FDNB-treated sample was passed through a nylon powder column to separate ε-DNP-lysine from a number of interfering compounds. Blom and his associates outlined three detection procedures for determination of DNP-lysine:

a) Direct photometry of the yellow colour.
b) Photometry of the reaction product with ninhydrin.
c) Polarographic measurement based on reducing the nitro group at the dropping mercury electrode.
In the original Silcock method the separation of ornithine and lysine was poor, thus causing some error in the estimation of lysine. Williams (1967) reported that the small shoulder on the lysine peak could be totally resolved by lengthening the column, although a higher pressure would then be necessary to maintain a sufficiently rapid flow rate. In cases where the ornithine peak is small and not clearly separated from the lysine peak, but is clearly distinguishable as part of it, it is possible to compute the true area due to lysine. Sometimes, however, the ornithine level is high in comparison to the area of the residual lysine peak, and so there could be a considerable error in the estimation of the true available lysine of the analyzed sample.

Ostrowski et al. (1970) used a modified short column technique to improve the resolution of ornithine and lysine. By using their technique, no automatic analyzer is required to determine total and unavailable lysine.

The DNFB Difference technique published by Couch in 1975 has been adopted as official-AOAC first action. The technical advantages of the Difference technique are that N-terminal lysine and free lysine are included, and that there is no problem from partial reduction of DNP-lysine during hydrolysis. The disadvantages are that lysine is more difficult to estimate than DNP-lysine unless one has
automated ion-exchange chromatography equipment, and that FDNB is not water soluble and has a vesicant effect on human skin.

2. The trinitrobenzene sulfonic acid method (TNBS)

A simpler test based on the same principle as that of Carpenter (1960) in which use is made of the reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS), instead of 1-fluoro-2,4-dinitrobenzene (FDNB), has been suggested by Kakade and Liener (1969). The reaction of TNBS with an amino acid is illustrated in Figure 3.

The original work with this compound on pure amino acids and peptides was carried out by Okuyama and Satake (1960) and Satake et al. (1960). The TNP-derivatives of amino acids and peptides were characterized by paper chromatography and spectroscopy. No reaction was observed with the side chains of histidine, tyrosine, threonine and serine after treatment for three days at elevated temperatures. This favourable specificity of TNBS prompted its use in the modification of cytochrome c (Takemori et al., 1962), haemoglobin (Shinoda, 1965), and xanthine oxidase (Greenlee and Handler, 1964). The SH groups of cysteine and mercaptoethanol were observed to react with TNBS (Kotaki et al., 1964). Goldfarb (1966) described the reaction of TNBS with human serum albumin and analyzed its course in terms of three classes of amino groups of different reactivities.
FIGURE 3. Reaction of 2,4,6-trinitrobenzene sulfonic acid (TNBS) with an amino acid. (From Hall et al., 1973).

\[
\text{2,4,6-Trinitrobenzene sulfonic acid} \quad \text{+ Amino acid} \xrightarrow{\text{NaHCO}_3 + \text{HCL}} \quad \text{Trinitrophenylated amino acid}
\]
The same reagent was used to establish the functional role of amino groups in some natural trypsin inhibitors (Haynes et al., 1967) with a mathematical procedure suggested by Ray and Koshland (1961).

Freedman and Radda (1968) examined quantitatively the reactivities of a variety of amino acids and peptides towards TNBS. Kinetic studies were performed by monitoring the extinction of the -NH-TNP group at 340 nm on a recording spectrophotometer. In agreement with Goldfarb's results they found that the reaction of the TNBS with amino acids and peptides is a second order reaction. The pH dependence showed that only the unprotonated amino group is the reactive species. The SH group of N-acetylcysteine was found to be more reactive to TNBS than most amino groups. However, the -S-TNP group is unstable at alkaline pH and has a much lower extinction coefficient at 340 nm than the -NH-TNP group. It was also found that both amino groups of lysine react with TNBS. However, at pH 7.4 and at room temperature, the ε-amino groups were 30 times more reactive to TNBS than the α-amino groups.

By using TNBS, Habeeb (1966) was able to study the reaction of free amino groups in protein with sodium dodecyl sulfate, potassium thiocyanate and formaldehyde. It was found that proteins such as bovine serum albumin, ovalbumin and human gamma-globulin have the property of binding
sodium dodecyl sulfate through the ε-amino group. The amino group involved at this binding site became unreactive to TNBS. Partial reaction of potassium thiocyanate with the free amino groups in bovine serum albumin, ovalbumin, human α-globulin and lactalysate was demonstrated by reaction with TNBS. It was also found that the reaction of bovine serum albumin with formaldehyde involves the formation of methylene cross links between the amino group on one hand and the amide, guanyl, indole, and imidazole groups on the other hand. The method developed by Habeed suffers the disadvantage that it does not differentiate between free ε-amino and N-terminal amino groups of proteins. This could be critical in the case of peptides, low molecular weight proteins such as insulin, or proteins containing several peptide chains or subunits.

Kakade and Liener (1969) developed a method to determine specifically the available lysine content of protein foodstuffs. The specificity of the technique for the ε-amino groups of proteins resides in the fact that, subsequent to acid hydrolysis of the TNP-protein, the α-TNP amino acids may be extracted with ether, whereas ε-TNP-lysine remains in the aqueous phase where it is determined spectrophotometrically. A hydrolysis period of 1 hr at 120°C with 6 N HCl seems sufficient for quantitative recovery. Complete hydrolysis of the protein is not necessary since small
TNP-peptides possess essentially the same solubility and spectrophotometric properties as the free TNP-amino acids. The amount of ε-TNP-lysine is calculated from a standard curve obtained with various levels of ε-TNP-lysine which has been subjected to the same procedure as the proteins. Kakade and Liener found close agreement between the available lysine values determined with TNBS and the values obtained with the DNFB method of Carpenter.

Holsinger et al. (1970) used the TNBS method of Kakade and Liener to evaluate the available lysine of acid precipitated caseins. αs1- and β-casein yielded available lysine amounts consistent with total lysine. However, available lysine of the κ-fraction was 15% higher than the total lysine content. An explanation for these high values could be the presence of D-glucosamine and D-galactosamine, and probably other uncharacterized glycoprotein fragments. Amino sugars react with TNBS to form colored derivatives which are not removed by ether extraction. Holsinger et al. concluded that although exact values of available lysine could not be determined by TNBS in foods containing large amounts of glycoproteins, the procedure could still find application in the determination of relative values of lysine inactivation during processing of products of identical composition.
Posati et al. (1972) noticed that the presence of lactose in cheese whey interfered with the determination of available lysine in whey protein when TNBS was used. Loss of ε-TNP-lysine was dependent on the amount of lactose in the test material. The sample weight used for analysis, also influenced the results. The larger the sample of either pure protein or carbohydrate containing material, the smaller the amount of available lysine found.

Hall et al. (1973) proposed a method for the determination of available lysine with TNBS in animal proteins, which differs in several aspects from that of Kakade and Liener. The necessity of accurate weighing of a very small sample, was eliminated by the use of agar suspensions of the finely ground material. They also modified the reaction conditions used by Kakade and Liener (0.1% TNBS solution for 2-hr at 60°C; 2-hr hydrolysis period with 6 N HCl in an autoclave at 120°C) and suggested, 0.5% TNBS solution reacting with the protein for 75 min at 40°C, followed by a 2-hr hydrolysis with 11 N HCl in a boiling water bath. The boiling water bath overcomes the serious corrosive action of the HCl upon the metallic interior of the autoclave. Their results for available lysine in animal protein concentrates were in close agreement with those measured by the Carpenter procedure.

Hall et al. (1973) in agreement with Ousterhout and Wood (1970) proposed the use of pure lysine as a standard instead of ε-TNP-lysine, since it has been shown that
\(\alpha, \varepsilon\)-diTNP-lysine initially formed is unstable in hot 6N HCl and is easily converted into \(\varepsilon\)-TNP-lysine (Kotaki and Satake, 1964).

Hall et al. (1973) investigated the reaction of TNBS with hydroxylysine, cadaverine and ornithine, and found that these compounds react to give TNP-products that remain in the aqueous phase after ether extraction and have absorbance values similar to \(\varepsilon\)-TNP-lysine.

TNBS is seriously affected by the presence of carbohydrate, partly because lysine is rapidly coupled with aldose groups, thus making the terminal amino linkage unavailable to form \(\varepsilon\)-TNP-lysine, and partly because of the adsorption of the \(\varepsilon\)-TNP-lysine on to the carbon particles (from charring) and to the interfering absorbance of the browning products. In consequence, false high levels of available lysine can be found in samples containing carbohydrate compounds. Furthermore, the amines agmatine, spermine, spermidine and taurine found in numerous plant species (Smith, 1972) could explain why the values for available lysine in certain samples are higher than the total lysine content (Hall et al., 1975).

Hall et al. (1975) concluded that TNBS can be used for determining available lysine in carbohydrate-rich material, provided that the sample mass does not exceed 5 mg and that the reaction is maintained for only 30 min at 30°C, instead of 75 min at 40°C. Under these conditions
the 'blank' absorbance, i.e. the value of the unreacted sample simply heated with HCl is very high in relation to the absorbance of the actual ε-trinitrophenylated product. Attempts to reduce this value to an analytically more acceptable level have had limited success.

Eklund (1976) slightly modified the method of Kakade and Liener (1969) by increasing the sample size as well as by subjecting the TNP-proteins to hydrolysis at 110°C for 90 min. Eklund determined available lysine in casein and rapeseed with his procedure, that of Kakade and Liener and the method of Rao et al. (1963). Throughout, he obtained higher values with his modified method, but the degree of variability between repeated analyses of the same material was lower as compared to the variability obtained with the method of Kakade and Liener.

3. Guanidination

Due to the relative instability of ε-DNP-lysine upon acid hydrolysis (Carpenter, 1960), Mauron and Bujard (1963) suggested the use of O-methyl-isourea for determination of available lysine. The guanidation with O-methyl-isourea transforms the ε-amino group of lysine into a guanidine derivative, which on hydrolysis yields homoarginine. The hydrolysate is analyzed by column chromatography and homoarginine is eluted with the basic amino acids, where it appears after arginine. No homoarginine is formed when the lysine units are combined with sugar in a Maillard reaction.
Although methyl-isourea appears to be a very specific reagent for the ε-amino groups of lysine, probably, the long reaction time (3 days) needed has precluded any extensive use of the approach.

4. Acrylonitrile

Acrylonitrile will also react, though again slowly, with the ε-amino groups of lysine to form a cyanoethylated derivative that is stable under the ordinary conditions for the acid hydrolysis of the proteins (Riehm and Scheraga, 1966). When the reagent was used by Pisano et al. (1968) and by Harding and Rogers (1971) to measure the reactive lysine units in cross-linked fibrin and hair proteins, the measurements agreed with the values for ε-N-(γ-glutamyl) lysine obtained by enzymic digestion in vitro.

5. Methyl acrylate

Methyl acrylate is an alternative to acrylonitrile (Cavins and Friedman, 1967), and its use for nutritional studies has been proposed by Finley and Friedman (1973).

The methyl acrylate method is based on condensing an α, β-unsaturated compound with available amino groups. The reaction of the accessible amino groups (non-terminal) of lysine in proteins with excess methyl acrylate at pH 9.1 yields, after hydrolysis, mainly ε,ε,N,N'-dicarboxyethyl-lysine and a small amount of ε,N-monocarboxyethyllysine. The
reaction time can be cut down to 4 hr by using a 75% DMSO (dipolar aprotic solvent): 25% (v/v) pH 9.1 Buffer. Available lysine is measured by comparing lysine content before and after alkylation.

Finley and Friedman reported good agreement between the methyl acrylate method and TNBS and DNFB procedures on a series of pure proteins.

6. Ethylvinyl sulfone

The ethylvinyl sulfone method (Friedman and Finley, 1975) may be used to measure nutritionally available lysine as the mono- and disubstituted ethylsulfonylethyl derivatives of lysine and also unavailable lysine that is liberated during acid hydrolysis as lysine remaining after ethylvinyl sulfone treatment. Available lysine is measured as the difference between the lysine content of the starting material before and after ethylvinyl sulfone treatment.

In principle, ethylvinyl sulfone can alkylate all functional groups containing active hydrogens in proteins; for instance, the ε-amino group of lysine, side chains and imidazole groups of histidine residues, by Michael-type nucleophilic addition reactions.

7. 2-Chloro-3,5-dinitropyridine

Selim (1965) employed Sanger's reagent 1-fluoro-2,4-dinitrobenzene (FDNB) to estimate the lysine content of protein hydrolyzates after blocking the α-amino groups of the free amino acids with copper.
A similar principle was used by Tsai et al. (1972) for the screening of lysine content in maize seeds. In their method the defatted protein sample is hydrolyzed with pronase or a mixture of alcalase and pancreatic trypsin, to yield free amino acids or low molecular weight peptides. The α-carboxyl and α-amino groups of the free amino acids are blocked with cupric ions (a copper phosphate suspension), leaving the ε-amino groups of lysine free to react. An ε-dinitro-pyridyl derivative of lysine is then formed on reaction with 2-chloro-3,5-dinitropyridine. Extraction of the reaction mixture with ethyl acetate removes the excess 2-chloro-3,5-dinitropyridine, leaving an aqueous solution of ε-DNPyr-lysine. The absorbance of this solution is then determined at 400 nm.

Lysine content as estimated by this colorimetric method is usually higher than the estimate of lysine content from the amino acid analyzer, but it is in good general agreement. The higher lysine values found in the colorimetric assays may be a consequence of the relatively minor contribution made to absorbance by the arginine present.

8. Sodium borohydride

Thomas (1970) used NaBH₄ for the determination of the available lysine content of cottonseed meal, a material in which lysine units may combine with the aldehyde groups of
the gossypol pigment, unless processing is carefully controlled.

Maillard-type compounds of aldehyde and lysine units break down on acid hydrolysis, to give a variable, but often high recovery of lysine. However, when they are treated with sodium borohydride they are reduced to acid-stable compounds. Thus, when proteins are treated with formaldehyde followed by borohydride treatment, ε-N,N-dimethyl-lysine units are formed (Means and Feeney, 1968). The 'total' lysine released on acid hydrolysis following such treatment may, therefore, be a measure of those lysine units in a test material that had not reacted with aldehydes.

Thomas (1970) and Couch and Thomas (1976) reported close agreement between available lysine determined by the FDNB-Difference technique and the sodium borohydride method. The chemical data compared very favorably with the data from chick assay.

The advantage of the sodium borohydride method, as compared with the official DNFB-Difference technique, is that it eliminates the necessity of making two hydrolyses and two determinations on the amino acid analyzer in order to obtain the available lysine data.

9. $^{19}F$ NMR

Ramirez et al. (1975) have developed a method to determine the ε-amino group of lysine by F NMR spectroscopy. Free ε-amino groups of lysine are trifluoroacetylated with
the reagent S-ethyl trifluorothioacetate in dimethyl sulfoxide solution and the number of such groups is quantitatively determined using standard $^{19}$F NMR technique.

Dimethyl sulfoxide is used as the reaction medium in order to improve the homogeneity of the system. When the mixture becomes homogeneous, a portion is added directly to an NMR sample tube and the $^{19}$F NMR spectrum is obtained. A typical spectrum is shown in Figure 4.

The resonance at lowest field (A) corresponds to trifluoroacetic acid, a hydrolysis product of the thiol ester. The peak at intermediate field (B) corresponds to the trifluoroacetylated $\varepsilon$-amino groups of lysine, and the peak at highest field (C), to the trifluoroacetyl group of the thiol ester (unreacted reagent).

The relative integrated intensities are calculated from the weights of the appropriately cut-off peaks. The molar ratio of lysine to the original thiol ester is given by the ratio of the weight of the intermediate peak to the total weight of all peaks.

The molar optimum ratio of thiol reagent to lysine is 4:1. Higher concentrations of thiol reagent lead to double substitution of some lysine sites. With lower concentrations, not all the $\varepsilon$-amino groups are attacked.

Excellent agreement was found between the compositional data for pure milk proteins and the lysine content determined with $^{19}$F NMR.
FIGURE 4. $^{19}$F NMR spectrum of the reaction products of a protein and S-ethyl trifluorothioacetate in dimethyl sulfoxide solution.
F NMR spectroscopy offers a reasonably accurate, fast and relatively simple procedure, without the difficulty of variability of results from varying amounts of protein present. It does require access to a $^{19}$F NMR spectrometer and fairly concentrated protein solutions.

10. The sodium dinitrobenzene sulfonate method (DNBS).

The DNBS technique for determination of available lysine, unlike most of the chemical methods so far described (2-chloro-3,5-dinitropyridine and $^{19}$F NMR are the exception), does not rely on acid hydrolysis of the treated protein in order to determine available lysine.

Previous methods for lysine determination using unhydrolyzed proteins include the Van Slyke nitrous acid method (Van Slyke, 1911, 1912) and the Hofman reaction and iodometric back titration method (Baraud, 1957). These methods however were not specific for the ε-amino group of lysine.

The work of Eisen et al. (1953), Li (1956), Ikenaka (1959), Hosoya (1960) and Sugae (1960) suggested that dinitrobenzene sulfonate could be used as a specific reagent for determining lysine in proteins without hydrolysis. Eisen et al. (1953) demonstrated that DNBS reacts specifically with only amino groups. Li (1956) reacted insulin with DNBS for 216 hr at 3°C in 0.1 M sodium carbonate and found that the ε-amino group of lysine was the only reactive
species; N-terminal glycine and phenylalanine, and the residue tyrosine did not react with DNBS. When DNBS was reacted with takaamylase-A at pH 10.7, 37°C, only 11 of 22 lysine residues in the enzyme molecule were dinitrophenylated after 50 hr (Ikenaka, 1959). Similarly, 3 out of 25 lysine residues in bacterial amylase reacted after 74 hr (Sugae, 1960). The calcium acetate, used to minimize denaturation of the enzyme, and the low pH used by these authors could render many of the lysine residues unreactive by steric and electrostatic factors.

Concon (1971) confirmed the validity of DNBS as a reagent for lysine determination in unhydrolyzed proteins, by reacting purified proteins with DNBS. The number of lysine residues per mole of protein was in full agreement with the reported values.

Concon attempted to speed up the DNBS reaction by increasing the pH, temperature and reaction time. It became evident that the difference in reaction rate between the α- and ε-amino groups of lysine is such that reaction conditions could be chosen in which only the ε-amino group will react. The optimum combination of factors for significant reaction of the ε-amino group with DNBS, lay within the following limits: pH 9.0 - 13.0; temperature 30 - 60°C; reaction time 0.5 - 2.0 hr. The reactivity of the ε-amino groups with DNBS, increased almost linearly with pH at 40°C. No reaction was observed at pH 9. The maximum reactivity
appeared to be between pH 12 and 13. At pH above 12, the reactivity of the α-amino group was apparently absent due to instability of the α-NH$_2$-DNP derivative. ε-NH$_2$-DNP derivative was stable at pH 12 even after 3 hr at 40°C. There was no advantage to be gained in terms of specificity by increasing the temperature to 45°C and at the same time decreasing the pH to 11. With most proteins a workable combination of pH 12.3, 40°C and 1 hr reaction time (pH 10.5, 60°C, 1 hr for rice) gave quite satisfactory results. These values were neither optimum nor ideal, but with pure lysine these conditions gave absorbance readings with no detectable contribution from the α-amino groups.

The specificity of the DNBS under the aforementioned conditions, was shown by paper chromatography of the acid hydrolyzed, DNBS-treated proteins. Only ε-N-DNP-lysine was detected (Concon, 1975a). Evidence of specificity was also obtained from a comparison of the specific reaction rate constants for pure lysine and cereal proteins. For grains analyzed at pH 12.3, 40°C, 1 hr the average value of $k$ was $2.5 \times 10^{-3}$ min$^{-1}$. The same value was found for pure lysine. For rice, analyzed at pH 10.5, 60°C, 1 hr the average value of $k$ was $6.2 \times 10^{-3}$ min$^{-1}$, which was also exactly the value of lysine under these conditions.

Large positive errors (20-40%) result when lysine in rice is determined at pH 12.3 and 40°C. It is believed that this is due to the high arginine content of rice. Under
high alkaline conditions and elevated temperature, arginine may degrade to ornithine. Ornithine (pK$_3$ = 10.8) reacts with DNBS similarly as lysine (pK$_3$ = 10.5). In addition, arginine with a pK$_3$ = 12.5 may react significantly with DNBS at pH 12.3. Lowering the pH to 10.5 apparently minimized these reactions, even at 60°C. The drop in pH necessitated a change in sulfhydryl masking agent to mercuric chloride (HgCl$_2$) since phenylmercuric chloride (PMC) precipitates below pH 12. Other grains give lower lysine values, by as much as 30-40%, when analyzed under the same conditions used for the rice samples. It is possible that because of the high arginine content, rice proteins have internal conditions alkaline enough to permit more complete dissociation of the ε-amino groups even though the pH of the surrounding solution is lower than that permitted by the Henderson-Hasselbach equation.

At pH 12.3, 40°C, 1 hr, the DNBS reaction is incomplete; only about 14% of the total lysine is reacted. However, it is possible to determine directly the amount of lysine from the fraction reacting in 1 hr. Kinetic studies indicate that in the presence of excess DNBS, the reaction follows first order kinetics as described by equation 1. The fraction of lysine reacting at any given time is directly proportional to the total lysine originally present.

\[
Lt = \frac{([e^{kt}-1] / e^{kt})}{L_0} \quad \text{(Equation 1)}
\]
where \( L_t \) = the fraction of lysine reacting at time \( t \); 
\( L_0 \) = the total lysine originally present; \( k \) = the specific reaction rate constant; and \( e \) = the base of the natural logarithm. \( L_t \) is directly proportional to \( L_0 \) at any given time. Therefore, from the absorbance due to \( L_t \), \( L_0 \) may be determined directly. A plot of the absorbance due to \( L_t \) versus \( L_0 \) conforms remarkably to Beer and Lambert's law.

Since the DNBS reaction is highly dependent on a direct proportionality between total lysine and the fraction reacting in any given time, every protein-bound \( \varepsilon \)-amino group must be equally accessible and reactive. These requirements are satisfied by conducting the dinitrophenylation reaction at high pH and in high concentrations of urea.

The DNBS reaction determines the \( \varepsilon \)-amino group of lysine in unhydrolyzed proteins. On the other hand, free amino acids may also react with DNBS. Tryptophan and cysteine are among the most reactive. Cysteine is seven times more reactive than free lysine because the SH group also reacts with DNBS. The DNBS derivatives of free amino acids, except for cysteine, arginine and histidine, can be removed by ether extraction. A major portion of the cysteine interference is eliminated by ether extraction. However, the sulfhydryl group should be masked by mercuration with PMC or \( \text{HgCl}_2 \). The slight reactivities of the other basic amino acids are reduced by mercuration. Mercury forms
complexes with basic amino acids in alkaline medium (Kai, 1967).

The reactivities of arginine and histidine are quite small when compared to lysine. Their interference can be eliminated to a certain extent with mercuric chloride (but not PMC) and by use of a smaller sample size. When the ratio of free arginine or histidine to lysine approaches 1:2, unreliable results become more evident even with the use of mercuric chloride.

The N-terminal amino groups of peptides are even less reactive to DNBS than the α-amino groups of free amino acids. For example, the reactivity of tryptophan as tryptophanyl-tryptophan is reduced by as much as 93%. In proteins, at pH 12, 40°C, 1 hr reaction time, the N-terminal amino group is virtually unreactive to DNBS.

Once reacted with DNBS reagent, the test protein is subjected to ether extraction, and the optical density is measured at 385 nm. The maximum wavelength of ε-DNP-lysine is between 360 and 365 nm. Unfortunately, the components of the reaction media (PMC, DNBS, phosphates) and the various cereal pigments, absorb strongly at these wavelengths.

The DNBS method appears to be rapid and sensitive for the ε-amino groups of lysine, with the advantage that it does not require acid hydrolysis of the proteins like most of the other chemical methods used for determination of lysine availability.
11. Fluorescamine

Purcell et al. (1976) have developed a rapid method of the determination of free amino groups in intact pure proteins, where protein hydrolysis and extraction of a labeled lysine derivative is not necessary. The method requires only two steps: labeling the primary amino groups with 4-phenyl-spiro-[furan-2-(3H)-1'-phthalan]-3,3'-dione, commonly referred to as fluorescamine, and measuring the labeled groups by absorption spectroscopy in the range 375-390 nm.

Fluorescamine, first synthesized by Weigele et al. (1972), reacts specifically with primary and secondary amino groups to yield a fluorescent and a nonfluorescent product respectively.

The result of reacting fluorescamine with proteins yields a direct estimate of the unsubstituted ε-amino groups and hence, of the available lysine content of proteins. However, the α-amino groups of the protein chain is also labeled, and is a minor contribution to the results obtained.


Dye-binding procedures are rapid, inexpensive methods which have been successfully adapted to automatic and semi-automatic systems of analysis.

Three classes of dyestuffs have been used for protein quality evaluation: phthalein dyes, reactive dyes and acid azo dyes (Lakin, 1973). Ney and Wirotama (1970) have reported
the use of the reactive dye, remazol brilliant blue R for the determination of available lysine in milk and cheese. The dye, whose structure is shown in Figure 5, when converted to the vinyl (active) form by heating in strong alkali, will form a covalent bond with the free ε-amino group of the lysine residues and with the thiol group of cysteine. No reactions were observed with the hydroxyl groups of serine and tyrosine. The authors claimed that reaction with cysteine can be disregarded in the case of milk proteins.

For determining available lysine the protein is reacted with remazol brilliant blue R, and the mixture is passed over Sephadex G-25 coarse. The dyed protein is eluted after 10 minutes and discarded. The unbound dye is eluted over the next 2 hr and its concentration is determined spectrophotometrically. The amount of bound dye is calculated by comparing the 280 nm extinction of the eluate with that of different solutions of the original activated dye solution in pH 8 buffer. This value was found to decrease with time when milk was maintained at 95°C; that is, the milk proteins bound less dye because of their content of available lysine fell.
FIGURE 5. Structure of the reactive dye remazol brilliant blue R. (From Ney and Wirotama, 1970).
Although Pruss and Ney (1972) demonstrated no interference from lactose and other sugars, the method suffers from the disadvantage that the reaction is dependent on the quantity of protein present in the sample. As the quantity of protein increases, the amount of dye bound decreases.

Most of the dye-binding results reported in the literature are given in terms of "dye-binding capacity" or "DBC". DBC is an almost pseudo-scientific term which implies that a given amount of protein has an affinity for a given amount of dye. This value depends upon the experimental conditions, the structure of the dye molecule, the composition of the buffer system and the purity of the dye. The temperature of the system and its pH are also important factors. Therefore, unless these parameters are specified, a statement of dye-binding capacity has no real meaning and may even prove to be misleading (Lakin, 1973).

Although quantitative determination of available lysine is difficult with this technique, dye-binding procedures could be used to monitor losses of lysine during the processing of food proteins, as long as the protein content of the material remains constant.

13. Chemical methods in the determination of available lysine in materials that have undergone Maillard reactions.

The type of damage which some of the chemical procedures fail to measure adequately is that occurring in
materials where lysine and reducing sugars have been in prolonged contact at relatively low temperatures (37°C), and in which 'early' Maillard compounds, of which α-N-formyl-(ε-N-deoxyfructosyl)-lysine (FFL) is a model (Finot and Mauron, 1972), would be expected.

Finot and Mauron (1972) reacted deoxy-ketosyl derivatives of lysine with FDNB (Carpenter, 1960), with TNBS (Kakade and Liener, 1969), and with O-methyl-isourea (Mauron and Bujard, 1963). When Carpenter's method was applied to FFL it was found that 14-28% of this unavailable form was determined as available lysine. Guanidination using O-methyl-isourea was the most specific (0%), whereas the TNBS method was completely non-specific (82-89%). Presumably, TNBS reacts with the basic secondary amine groups of the early Maillard compound and then on acid digestion the presence of the TNP groups weakens the sugar linkage which splits off to give a high yield of TNP-lysine.

O-methyl-isourea appears to be a highly sensitive indicator of all types of lysine binding, including the formation of early Maillard compounds. Although it cannot be recommended in its present form as a routine quality-control measure because of the time taken (3 days) for an individual determination, and also because different pH values are needed for different materials, it is of special interest
as a reference method.

When FFL was subjected to FDNB-difference treatment (Finot and Mauron, 1972; Hurrell and Carpenter, 1974), total lysine minus FDNB-reactive lysine was considerably greater than available lysine. This finding showed that FDNB would still react with a lysine unit in which the epsilon amino group was bound to a sugar. The FDNB-lysine-sugar complex breaks down on acid hydrolysis to yield neither lysine nor DNP-lysine to any significant extent.

It can be concluded that Carpenter's FDNB, O-methyl-isourea and sodium borohydride methods (Hurrell and Carpenter, 1974) give a good estimate of the available lysine in 'early Maillard' damage as seen in roller-dried milk powders (Mottu and Mauron, 1967). With 'advanced Maillard' and protein-protein damage, where there is a decrease in overall protein digestibility, these procedures may still overestimate available lysine, but they are, of course, more sensitive indicators of damage than is total-lysine determination.

Finot (1973) suggested a method for the evaluation of available lysine in heat-damaged milk powders based on the determination of total lysine and furosine content measured after acid hydrolysis of the protein. When pure ε-N-deoxy-ketosyl-lysine derivatives are hydrolyzed, three main compounds are obtained: lysine, furosine and pyridosine.
These compounds are always present in the same proportions, the result being dependent only on the concentration of the acid used for hydrolysis (Finot and Mauron, 1972). The quantity of lysine blocked in the deoxy-ketosyl form, that is, unavailable lysine, can be calculated directly from an acid hydrolysate of the protein.

Hurrell and Carpenter (1974) reported that autoclaving protein with sucrose for 2 hr resulted in less furosine production than did 1 hr. Presumably this is because the more complex polymers formed in the advanced stages of the Maillard reaction do not break down to furosine on acid hydrolysis. Sulser (1973) has reported similar results. While the presence of furosine in the acid-hydrolysate of a test material indicates that some of its lysine has been involved in Maillard reactions, it cannot be used as a quantitative indicator in all instances of protein-sugar damage.

D. ENZYMATIC METHODS

Even though the amino acid profile is important in evaluating the nutritive quality of a protein, the digestibility of that protein is the primary determinant of the availability of its amino acids. The digestibility of a food protein may be obtained by using a rat bioassay but this is an expensive and time consuming procedure.

Several in vitro-enzymic digestion methods for the measurement of protein availability have been developed. One of the earliest procedures was that of Melnick et al.
In his procedure food proteins were digested with pancreatin. At intervals, aliquots of the incubation mixture were withdrawn and the degree of hydrolysis was measured by formol titration. It was found that those factors known to increase the nutritive value of soy protein also increased the susceptibility of the protein to enzymatic digestion. It was also found that methionine is released earlier from heat processed soy meal than from raw soy meal.

Melnick and co-workers (1946), and Riesen et al. (1947) proposed that in addition to the total amino acid composition, the rate of release of amino acids from protein by pancreatic digestion was an important factor in the nutritional quality of a protein. This concept was utilized by Horn et al. (1953) to evaluate the nutritional quality of food proteins by measuring microbiologically the individual amino acids made available by pepsin, trypsin, and hog mucosa. This method gave good correlation with the biological value of cottonseed meal which had been subjected to various degrees of processing; however, there was no indication that it could be used to compare proteins from different sources.

Evans and Butts (1948) found that enzymic hydrolysis of soybean protein brought about some loss of all essential amino acids, in the sense that they had been rendered unavailable for liberation by digestive enzymes, though they were evidently regenerated by acid hydrolysis. When heating in the presence of sucrose, large amounts of the basic and sulfur amino acids had become unavailable and the losses
were much greater than in the absence of sucrose.

Sheffner et al. (1956) by combining the pattern of essential amino acids released by in vitro pepsin digestion with the amino acid pattern of the remainder of the protein, described an integrated index, the Pepsin-Digest-Residue (PDR) amino acid index. Division of the PDR index by the digestibility coefficient of the respective proteins, yielded values which accurately predicted the biological value of the proteins studied.

Since enzymatic hydrolysis may be inhibited by the hydrolysis products which in vivo are rapidly absorbed, dialysis (Mauron et al., 1955) and gel filtration (Ford and Salter, 1966) have been used to remove the amino acids as they are released. Mauron et al. (1955) employed an in-vitro digestion procedure in which the test protein was simultaneously digested and dialyzed, first with pepsin and then with pancreatin. Although the method has been employed occasionally for other food proteins, it has been used extensively in the quality control of heat processed milk. In milk, the loss of lysine availability is due to a Maillard type reaction of the lysine side chain with lactose. This linkage at the ε-amino group is resistant to the action of the digestive enzymes. A comparison of the values for available lysine obtained by this enzymatic procedure with those achieved with Carpenter's chemical method (1960) and
those from biological evaluation based on growth, showed good conformity between the three methods, on a series of milk samples (Bujard et al., 1967; Mottu and Mauron, 1967).

Ford and Salter (1966) attempted the continuous removal of reaction products from the in vitro digestion system, by causing the enzyme-substrate mixture to pass through a calibrated column of Sephadex gel G-25. Each digest was thus resolved into four fractions: the residue and three soluble fractions, 'soluble protein', 'peptide', and 'free amino acids'. Freeze-dried cod fillets subjected to different heat treatments were used as test proteins. Microbiological assay of all the fractions and the original test protein after prolonged digestion with pepsin, pancreatin and erepsin, gave broadly similar results. With increasing temperature, the 'free amino acid' component in the digests became increasingly deficient in several amino acids relative to their content in the original unheated meal, notably in lysine and the sulphur-containing amino acids.

Mauron (1970) calculated the in-vitro amino acid availability from the data of Ford and Salter, by dividing the value in the 'free amino acid' fraction of the heated samples by the corresponding value in the unheated sample. He found good agreement between his in-vitro digestion procedure after dialysis and the results of Ford and Salter.
Another way to separate the undigested residue from
the low molecular weight soluble fractions was experimented
by Prahl and Taüfel (1966). These authors replaced dialysis,
by filtration through a membrane under pressure. Amino acid
data are however not yet available with this method, since
only nitrogen was measured.

Akeson and Stahmann (1964) used pepsin followed by
pancreatin digestion to estimate the digestibility and
protein quality of processed foods. Upon digestion of the
protein, the undigested protein and peptides were precipita­
ted with picric acid and the amino acids were determined by
automatic amino acid analysis. Using whole egg as a
standard they found excellent correlation between the pepsin-
pancreatin index values for 12 proteins and the biological
values reported in the literature, from feeding trials.

In 1975, Stahmann and Woldegiorgis slightly modified
the procedure of Akeson and Stahmann by introducing sulfo-
salicylic acid, instead of picric acid, as a precipitant
of the undigested protein and peptides. Sulfosalicylic
acid is ninhydrin negative (Perry and Hansen, 1969) and can
be directly applied to the columns for amino acid analysis.

Menden and Cremer (1966) reported that hydrolysis by
pancreatin alone or in combination with acid hydrolysis
gives a better indication of protein quality of processed
casein and meat products than acid hydrolysis alone. They
subjected the protein to a short digestion time with a large
amount of pancreatin. The authors argue that the initial steps in digestion are of importance and that it is preferable to digest with a high concentration of enzyme for a shorter period, so as to avoid auto-hydrolysis of the enzymes used.

Buchanan (1969) and Buchanan and Byers (1969) described an in vitro system for measuring protein digestibility on a wheat leaf protein concentrate with an enzymatic digestion utilizing papain. Examination of the data shows a fair degree of correlation between the in vivo feeding trials and in vitro figures on a freeze-dried material, but a serious difference in the case of the same material after moist heat treatment. Buchanan was unable to obtain a correlation of protein digestibility by Akeson and Stahmann's procedure, and his results with papain showed poor correlation between in vivo and in vitro data.

Saunders et al. (1973) employed the pepsin-pancreatin system described by Akeson and Stahmann (1964), and the papain method of Buchanan and Byers (1969) to determine the protein digestibility of alfalfa protein concentrates. They found excellent correlation between the values obtained from the enzyme system used by Akeson and Stahmann and in vivo data (0.874), whereas very poor correlation was found between the papain digestion and in vivo data. The papain system may have produced low values compared to the in vivo data.
because of interference in the digestion by the high lipid level in alfalfa protein concentrates.

Saunders et al. (1973) developed a pepsin-trypsin system, the in vitro results of which correlated well ($r = 0.914$) with in vivo digestibility. After the pepsin-trysin digestion, the protein remaining insoluble was analyzed for nitrogen content and the protein digestibility was calculated as the difference between the nitrogen in the protein concentrate and the nitrogen in the undigested fraction.

Rayner and Fox (1976) have criticized the fact that most of the in vitro enzymatic methods do not result in maximum digestibility during enzymolysis, so that the determination of amino acids released from a protein is not an absolute method of measuring availability. A reference standard represented by an optimally treated test material is therefore necessary.

Rayner and Fox (1976) used the enzyme pronase to digest autoclaved rapeseed meals. After incubation, the undigested protein was precipitated with picric acid and the amino acids were determined on an amino acid analyzer. A correlation coefficient of 0.991 was obtained when comparing in-vitro available lysine with Silcock available lysine. The recovery of amino acids from the pronase in-vitro enzymolysis showed recoveries approaching 100% for most
amino acids, but low recoveries for cysteine, methionine and tryosine, probably due to oxidation by picric acid. An interesting result was the high recovery of tryptophan. Akeson and Stahmann (1964) reported that tryptophan is destroyed by picric acid, however these results indicated that tryptophan can be determined by this procedure.

The application of the pronase in-vitro procedure, also allows to discover the presence of trypsin inhibitors. Trop and Birk (1970) reported that 90% of natural trypsin inhibitors inactivate pronase.

The method developed by Dvorak (1968) also uses total enzymatic digestion to determine the maximum theoretical amount of available amino acids. Available essential amino acids from native and heat-treated beef serum albumin, in the presence of glucose or without it, were determined microbiologically, after total enzymic hydrolysis of the protein by papain, leucine aminopeptidase and prolidase. This enzyme combination was previously used by Hill and Schmidt (1962). In this system leucine aminopeptidase is used to hydrolyze all peptide bonds that remain intact in papain hydrolysates, except those which contain the imino nitrogen of proline. Proline peptides are hydrolyzed by prolidase. Results of this study showed that the availability of amino acids in native serum albumin, microbiologically determined after total enzyme digestion,
corresponds to the theoretical amount in the protein.

Maga et al. (1973) pointed out that the initial rates of hydrolysis by trypsin on some commonly used protein sources were good indicators of the protein digestibility. After incubation of the protein with trypsin, incubation mixtures were withdrawn at one minute intervals and the pH was immediately recorded. In all products, most of the proteolysis occurred during the first few minutes and then remained constant. Steaming of the products resulted in faster hydrolysis rates. It became apparent that although certain vegetable proteins may have a high nutritional and biochemical values, their digestive acceptability may be quite poor since they are not rapidly hydrolyzed in the digestive system.

Rhinehart (1975) modified Maga's procedure and examined several enzyme systems which included trypsin, pepsin-trypsin, trypsin-chymotrypsin, and trypsin-chymotrypsin-peptidase combinations. The results were encouraging, with correlation coefficients of 0.79, 0.72, 0.80 and 0.74, respectively.

These aforementioned in vitro methods have not been widely accepted, either because no corresponding in vivo data were presented or because the procedures were complicated and time consuming, consequently, difficult for application in
routine in-plant quality control.

Hsu et al. (1977) developed a multienzyme system for the estimation of protein digestibility. The method was based on the digestion of an aqueous protein suspension at 37°C, pH 8, using a combination of trypsin, chymotrypsin and peptidase. As the proteolytic enzymes attack and break the peptide bonds within the protein's primary structure, the freed carboxyl groups that formed, immediately released $H^+$, which, in turn, lowered the pH of the protein suspension. The pH drop was monitored with a recording pH meter. The pH of the protein suspension after 10 minutes digestion with the multienzyme solution was highly correlated with the in vivo apparent digestibility of rats.

Hsu et al. were able to demonstrate that the multienzyme technique was unaffected by food lipids and buffering salts commonly found in foods, and was sensitive enough to be able to detect the presence of soybean trypsin inhibitor in soy flours, and chlorogenic acid in leaf protein concentrate. The method was also sensitive enough to detect changes in protein digestibility that occurred during the heat processing of specific foods.

A multienzyme system could reduce the effect caused by a specific enzyme inhibitor. Consequently, using a combination of enzyme instead of trypsin alone, could avoid under-predicting the digestibility of proteins containing
trypsin inhibitor. A single enzyme system that attacks at a specific peptide bond may give different results for proteins containing different concentrations of the specific amino acid. A multienzyme system reduces the limitations that are evident for a single enzyme system, and gives a better approximation of protein digestibility. The most significant advantage of this in vitro method for predicting apparent protein digestibility is that it can be completed within one hour and with a high degree of sensitivity.
MATERIALS AND METHODS

A. Materials

β-lactoglobulin was obtained from Nutritional Biochemical Corporation, Cleveland, Ohio. It was crystal-
lyzed and lyophilized powder from milk. Salt-free lysozyme was purchased from Worthington Biochemical Corporation, Freehold, N.J. 1.5 N HCl solubilized gluten was prepared according to the method of Wu et al. (1976). Casein (pH 7.5) and dried whole egg solids were prepared in the laboratory. Ribonuclease-S-peptide (bovine pancreas, grade XII-PE, MW 2166.35), pancreatin (porcine pancreas, grade VI), and pepsin (hog stomach mucosa, 2x crystallyzed and lyophilized powder) were the products of Sigma Chemical Company, Saint Louis, Mo.

The amino acid derivatives N-ε-formyl-L-lysine (MW 174.2), L-lysyl-L-lysine dihydrochloride (MW 347.3) and L-lysyl-L-alanine dihydrobromide (MW 379.1) were from Sigma Chemical Company.

N-α-formyl-L-lysine (MW 174.2) was synthesized according to the method described by Hofmann et al. (1960). Acetic anhydride was added to a solution of L-lysine formate in 98% formic acid. L-lysine formate was prepared from lysine monohydrochloride with Amberlite IR-4B in the formate cycle. The solvents were evaporated. The resulting oil was dissolved in ethanol and the crystals were collected and
recrystallized from ethanol. The purity of the compound was assessed on an amino acid analyzer.

α- and ε-carbobenzoxy-lysines were obtained from Eastman Organic Chemicals, Dallas, Texas. ε-TNP-L-lysine. HCl. H₂O (MW 411.8) was from ICN Pharmaceutical Inc., Life Science Group, Cleveland, Ohio.

1-fluoro-2,4-dinitrobenzene (FDNB) was from Calbiochem, San Diego, CA. 2,4-dinitrobenzene sulfonic acid (DNBS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) in the form of trihydrate (80% min. anhydrous by titration) were obtained from Eastman Kodak Company, Rochester, N.Y. 5-sulfosalicylic acid (98%) was from Aldrich Chemical Company, Inc.

B. Nitrogen determination

The nitrogen content of β-lactoglobulin, lysozyme, casein, acid solubilized gluten and eggs was determined by the rapid micro-Kjeldahl procedure of Concon and Soltess (1973). The protein content was calculated by multiplying the nitrogen content by a factor of 6.25 for egg and lysozyme; 6.38 for casein and β-lactoglobulin; 5.7 for gluten.

C. Amino acid analysis.

The digested protein was analyzed on a single column system (Durrum Chemical Corporation, Palo Alto, CA) attached to a Phoenix Model M6800 Amino Acid Analyzer (Phoenix Precision Instrument Company).
D. Lysine determination by the dinitrobenzene sulfonic acid method.

The lysine availability of various proteins was determined according to the method developed by Concon (1971). The reagent 2,4-dinitrobenzene sulfonic acid is used to complex the free ε-amino groups of lysine.

Reagents

Reagent A

50 g of urea and 7 g of Na₃PO₄·12H₂O were dissolved in 36 ml of hot water with a magnetic stirring bar, then 100 mg of phenylmercuric chloride was added. The solution was allowed to cool and the volume was made up to 100 ml. The solution was filtered through Whatman No.1 and the pH was adjusted to 12.30 - 12.35 with 1N NaOH.

DNBS reagent

6 g activated charcoal was added to 6 g of DNBS powder dispersed in 100 ml of water. It was then stirred for 10 minutes and filtered through Whatman No. 1. The refractive index was measured and the solution was diluted to obtain the final concentration of 3% DNBS. The reagent was kept frozen in small vials. (See appendix 1).

Ether extractant

Equal volumes of peroxide-free ethyl ether and distilled water were mixed in an extraction funnel, allowing the gas to escape from time to time. The aqueous layer was
discarded and the water-saturated ether was used.

Other reagents and materials

90% formic acid; 0.075 N NaOH solution; boiling chips (Hengar granules, Hengar Co., Philadelphia, PA), 10 mesh. The chips were soaked in concentrated HCl for 30 minutes, washed with water and dried.

Lysine standard

2.5 mM lysine solution was prepared by dissolving 45.66 mg of lysine monohydrochloride (MW = 146.20) in distilled water and diluting to 100 ml. From this stock solution, five levels of lysine standards were prepared as follows:

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>2.5 mM lysine soln. (mls)</th>
<th>0.075 N NaOH (mls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>0</td>
</tr>
</tbody>
</table>

These dilutions contained 0.125, 0.250, 0.375, 0.500 and 0.625 micromoles of lysine per 0.25 ml, respectively. They were used along with each experiment for the construction of the standard curve. The dilutions were kept frozen in small vials.
Protein solutions

The test protein was dissolved in 25 mls of 0.075 N NaOH to give a final concentration range of 30 - 100 µg of lysine per 0.25 ml of protein solution. The mixture was shaken on a vortex mixer and centrifuged at 2,500 x g for 20 minutes. The supernatant was collected and used for analysis.

Preparation of working protein solutions

<table>
<thead>
<tr>
<th>Protein</th>
<th>Literature value</th>
<th>Desired level</th>
<th>mg protein in 25 ml 0.075N NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluten</td>
<td>1.6</td>
<td>40</td>
<td>250</td>
</tr>
<tr>
<td>Casein</td>
<td>7.6</td>
<td>76</td>
<td>100</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>5.7</td>
<td>57</td>
<td>100</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>11.3</td>
<td>56.5</td>
<td>50</td>
</tr>
<tr>
<td>Egg</td>
<td>6.2</td>
<td>62</td>
<td>100</td>
</tr>
<tr>
<td>RNase-S-peptide</td>
<td>10.3</td>
<td>51.5</td>
<td>50</td>
</tr>
</tbody>
</table>

Calculation for gluten

\[
\frac{100 \text{ mg gluten}}{1.6 \text{ mg lysine}} \times \frac{40 \text{ µg lysine}}{0.25 \text{ ml}} \times 25 \text{ ml NaOH} = 250 \text{ mg gluten diluted to 25 ml with 0.075 N NaOH.}
\]

Procedure

0.5 ml of reagent A was added to all tubes without touching the sides; 0.25 ml of protein solution was then pipetted to the surface of sample and sample blank tubes.
To the reagent blank, instead of protein, 0.25 ml of 0.075 N NaOH were pipetted; to the standards, 0.25 ml of two to three levels of lysine standard solutions were added to the surface of reagent A. The solution was mixed thoroughly by firm wrist motion, and allowed to stand for a few minutes. 0.25 ml of 3% DNBS was very carefully added to all tubes, except for sample blanks which received 0.25 ml of water. The DNBS was added, first to reagent blanks and then to standards and samples in increasing lysine concentration. After thoroughly mixing, the tubes were immersed in a water bath at 40°C and incubated for 1 hr.

The tubes were then cooled in an ice-water bath. 2.0 ml HCL was added to all tubes except for sample blanks, to which 1.75 ml HCL and 0.25 ml 3% DNBS were added.

Ether extraction was carried out three times with 3.0, 2.5 and 2.5 ml of water-saturated ether. A vortex mixer was used for effective blending and extraction. Each tube was shaken for a few seconds, to avoid the formation of a gel or emulsion at the ether phase. The ether layer was carefully removed with a Pasteur pipet by means of a gentle suction. A clean Pasteur pipet was used for each solution. 0.5 ml of 90% formic acid and one Hengar boiling granule were added to all tubes. The residual ether was eliminated in a water bath at 55°C for no more than 15 minutes. The solution was cooled down to room temperature and the absorbance of samples, sample blanks and standards were
measured at 385 nm in a Beckman Spectrophotometer DB against
the reagent blank. It was easier and more reliable to take
the absorbance readings on the following day, when the
droplets on the sides of the tubes had set down. In this
case the tubes were kept in a dark place to protect them
from light.

The flow diagram of the experimental procedure is
shown in Figure 6. The amount of εDNP-lysine was calculated
from the standard curve obtained with different levels of
lysine, as shown in Figure 7.

The same procedure was carried out on α- and
ε-formyl-lysines, α- and ε-carbobenzoxy lysines, ribonuclease-
S-peptide and the dipeptides lysyl-lysine and lysyl-alanine,
in an attempt to determine the specificity of the DNBS
method for the epsilon-amino group of lysine.

Calculation

Casein absorbance, ave. of 3 determinations = 0.300.
From the standard curve, an absorbance of 0.300 corresponds
to 0.450 μmoles of lysine/3 ml.

Available lysine content in casein is calculated as
follows:

\[
\frac{0.450 \, \text{μmoles lys/3 ml}}{0.0010 \, \text{g casein/3ml}} \times \frac{1 \, \text{mol}}{10^6 \, \text{μmol}} \times \frac{146.2 \, \text{g}}{1 \, \text{mol lys}} \times 100 \, \text{g casein} = 6.58 \, \text{g of lysine/100 g casein.}
\]
FIGURE 6. The dinitrobenzene sulfonate (DNBS) method for determination of available lysine.
FIGURE 7. Standard curve for available lysine determination for a sample treated with dinitrobenzene sulfonic acid.
For the other proteins, the amount of sample per 3 ml of solution was: 0.0005 g of β-lactoglobulin; 0.0010 g for eggs and lysozyme; 0.0025 g for gluten, and 0.00135 g for RNase-S-peptide.

E. Lysine determination by the trinitrobenzene sulfonic acid method.

The TNBS method to determine available lysine in proteins was derived from the procedure of Eklund (1976). The reagent 2,4,6-trinitrobenzene sulfonic acid is used to complex the free ε-amino groups of lysine. The scale of the experiments was reduced to one tenth of the original procedure.

Reagents

34 mM TNBS solution: 590.26 mg of 2,4,6-trinitrobenzene sulfonic acid (MW 347.21) were dissolved in distilled water and the volume made up to 50 ml. The solution was stored in a dark bottle and kept frozen.

0.48 M sodium bicarbonate solution: pH 8.5 and 8.07 g of sodium bicarbonate were dissolved in distilled water and diluted to 200 ml.

Water-saturated diethyl ether: was prepared as described for the DNBS technique.

Protein solutions

90 mg of test protein was dissolved in 15 ml of 0.47 M NaHCO₃, pH 8.5 so that the protein extract contained
6 mg protein per ml. The protein extracts were prepared fresh for each experiment, since the stability of the protein in sodium bicarbonate is unknown.

\textcolor{red}{\textbf{\textit{\epsilon-TNP-lysine standards}}}

62 mg of \textit{\epsilon-TNP-lysine-HCl.H_2O} were made up to 50 ml with 0.48 M \textit{NaHCO_3} solution. A series of tubes containing graded levels of \textit{\epsilon-TNP-lysine} were set up as follows:

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>\textit{\epsilon-TNP-lysine}/NaHCO_3 soln. (mls)</th>
<th>0.48 M NaHCO_3 (mls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.10</td>
<td>0.40</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
<td>0.30</td>
</tr>
<tr>
<td>3</td>
<td>0.30</td>
<td>0.20</td>
</tr>
<tr>
<td>4</td>
<td>0.40</td>
<td>0.10</td>
</tr>
<tr>
<td>5</td>
<td>0.50</td>
<td>0.00</td>
</tr>
</tbody>
</table>

These dilutions contained 0.301, 0.602, 0.903, 1.204 and 1.505 \textmu mol of \textit{\epsilon-TNP-lysine} per final volume of 25 ml. They were used for the construction of the standard curve.

\textcolor{red}{\textbf{\textit{Procedure}}}

0.5 ml of protein extract was placed in a hydrolysis tube and shaken in a water bath at 40°C for 10 minutes.

0.5 ml of 34 mM TNBS solution was added to the protein extract. To avoid crystallization of the supersaturated TNBS solution in the pipet, the solution was warmed to 40°C prior to use. Instead of TNBS, 1.5 ml HCL was added to the sample blanks.
After an incubation period of 2 hr at 40°C, the tubes were immersed in an ice-water bath, and 1.5 ml of concentrated HCL was added to samples; 0.5 ml of TNBS was added to sample blanks.

The tubes were sealed and the contents were hydrolyzed for 90 minutes in a Reacti-Therm heating module at 110°C. The tubes were allowed to cool at room temperature and the hydrolysates were filtered through Whatman 1 and diluted to 25 ml with distilled water.

5 ml-aliquots were transferred to stoppered test tubes and extracted four times with 5 ml of water-saturated ether. Residual ether was evaporated by placing the tubes in a shaking water-bath at 55°C until no ether smell could be detected.

The absorbance of the aqueous protein solution was measured at 346 nm in a Beckman Spectrophotometer DB against the sample blank.

The flow diagram of the experimental procedure can be seen in Figure 8.

The amount of ε-TNP-lysine (or its lysine equivalent) was calculated from the standard curve obtained with various levels of ε-TNP-lysine, measured at 346 nm, as shown in Figure 9.
FIGURE 8. The trinitrobenzene sulfonic acid method for the determination of available lysine.

Protein

<table>
<thead>
<tr>
<th>TNBS treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>α- and ε-TNP-protein</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Acid hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-TNP-amino acids</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>ε-TNP-lysine</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ether extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ether layer</td>
</tr>
<tr>
<td>Aqueous layer</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ether layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-TNP-amino acids</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Aqueous layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ε-TNP-lysine</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ε-TNP-lysine</th>
</tr>
</thead>
</table>

| Read at 346 nm |
FIGURE 9. Standard curve for available lysine determination for a sample treated with 2,4,6-trinitrobenzene sulfonic acid.
Calculation

Gluten absorbance, ave. of 3 determinations = 0.100. From the ε-TNP-lysine standard curve, an absorbance of 0.10 corresponds to 0.240 μmoles of ε-TNP-lysine.

Available lysine content in gluten is calculated as follows:

\[
\frac{0.240 \, \text{μmoles} \, \varepsilon\text{-TNP-lys.}/25 \, \text{ml}}{0.003 \, \text{g} \, \text{gluten}/25 \, \text{ml}} \times \frac{1 \, \text{mol}}{10^6 \, \text{μmol}} \times \frac{146.2 \, \text{g}}{1 \, \text{mol} \, \text{lys.}} \times 100 \, \text{g}
\]

= 1.17 g lysine/100 g gluten.

F. Available lysine determination by the dinitrofluorobenzene method.

The 'difference' technique described by Couch (1975) has been used to determine available lysine of proteins. This method has been adopted as official-first action, AOAC. The reagent 1-fluoro-2,4-dinitrobenzene (DNFB) is used to complex the ε-amino groups of lysine. Available lysine is calculated as the difference between total and unavailable lysines.

Reagents

The reagent DNFB, solid at room temperature, was warmed up prior to use.

10% NaHCO₃ solution: 2 g of sodium bicarbonate was dissolved in deionized water and the volume made up to 20 ml.

6 N HCL: 495.87 ml of concentrated hydrochloric acid (12.1 N) were diluted to 1 liter with deionized water.
Sodium citrate buffer pH 2.2: 137.26 g Na citrate 2H₂O was dissolved in water, and 26 ml HCl added. 40 g Brij-35 was dissolved in water by heating and added to the solution, followed by 0.4 ml n-caprylic acid. The solution was diluted to 4 liters and filtered.

Procedure

1. Preparation of protein hydrolysate with DNFB (Unavailable lysine).

0.1 g of protein was placed in a dry 2-liter boiling flask, with a few glass beads. Care was taken to deposit the sample at the bottom. 10 ml freshly prepared 10% NaHCO₃ solution was added, care being taken that no meal adhered to the sides of the flask. The contents were thoroughly mixed by gentle swirling and the suspension was allowed to stand for 10 minutes.

A solution of 0.4 ml DNFB in 10 ml ethanol was then added and mixed by gentle swirling. The side of the flask was rinsed with 3-5 ml ethanol. The flask was stoppered and the contents were shaken under subdued light for 3 hr on a mechanical shaker.

2 ml of 6 NHCl was added. The mixture was concentrated to oily dryness in a vacuum rotary evaporator at 40°C,
and the residue was extracted with four-50 ml portions of anhydrous ether. In each case, the ether was mixed thoroughly with the semisolid mass and the two phases were allowed to separate completely before the ether was decanted. Final traces of ether were removed by aeration in the rotary evaporator, without vacuum.

125 ml of 6 N HCl was added and the mixture boiled under reflux for 18 hr, maintaining a constant stream of prepurified nitrogen through a capillary tube which reached about 2.5 cm above the surface of the solution (Figure 10). The mixture was allowed to cool down to room temperature and the condensor was washed down with 6 N HCl.

If time permitted at the cooling stage, the flask was kept overnight in the refrigerator, since it helped to precipitate the excess DNFB and reduce the subsequent work of extraction (Carpenter et al., 1957).

The mixture was then evaporated to a sticky paste in a vacuum rotary evaporator at 50°C. The paste was washed by adding 100 ml of deionized water and drying back to a paste on the vacuum rotary evaporator at 50°C. This procedure was repeated 5 times. The last evaporation was carried on until the sample was completely dry.

100 ml of pH 2.2 sodium citrate buffer was added. The flask was stoppered, shaken for 5 minutes and filtered through Whatman 43 into a storage bottle that was kept in
FIGURE 10. Refluxing system used in the preparation of protein hydrolysates.
a cold room at 5°C; 1.0 ml filtrate containing 0.72-0.88 mg protein was used for amino acid analysis.

2. Preparation of protein hydrolysate without DNFB
(Total lysine)

0.025 g of protein sample was placed in a 2 liter round-bottom flask with 4-5 glass beads. 200 ml of 6 N HCl was added and the mixture allowed to boil on a heating mantle, until 100 ml HCl had evaporated.

The mixture was hydrolyzed under reflux for 24 hr, as described under preparation of sample with DNFB.

The hydrolysate was evaporated to sticky paste in a vacuum rotary evaporator at 50°C. It was washed 5 times with 100 ml-portions of deionized water and evaporated to dryness during the last evaporation.

After 100 ml of sodium citrate buffer, pH 2.2 was added, the contents were shaken and filtered through Whatman No. 43. 1.0 ml filtrate containing 0.18-0.22 mg protein was used for analysis on an amino acid analyzer.

The flow diagram of the experimental procedure is shown in Figure 11.

Calculation.

The peaks on the chromatograms were integrated by multiplying the height of the peak (absorbance reading at the maximum peak height, minus absorbance reading at the base-
FIGURE 11. The dinitrofluorobenzene method for determination of available lysine (difference technique).
From: Blom et al., (1967).

Protein

Acid hydrolysis

DNFB treatment

Column chromatography

Aqueous layer

Total lysine

\[ \text{Total lysine} - \text{Unreacted lysine} = \text{Available lysine}. \]
line of the peak) by the width at half the height (dot count). The area under the peak was then divided by the constant for the given amino acid so that, micromoles or micrograms of amino acid per volume applied to the analyzer, were obtained. This was very easily done by using a program on the Monroe calculator.

The values were adjusted so that the sum of all the amino acids was 100%.

Available lysine content in casein was calculated as follows:

1. Total lysine
   \[14.035 \mu g \text{ lysine/ml (volume applied)} = \]
   \[1403.5 \mu g \text{ lysine/100 ml/25 mg casein} = \]
   \[5.614 \text{ mg lysine/100 mg casein}.\]

2. Unavailable lysine
   \[2.632 \mu g \text{ lysine/ml} = \]
   \[263.2 \mu g \text{ lysine/100 ml/100 mg casein} = \]
   \[0.2632 \text{ mg lysine/100 mg casein}.\]

3. Available lysine = Total lysine - Unavailable lysine
   Available lysine = 5.3508 mg lysine/100 mg casein.

G. Enzymatic digestion test

The digestibility of proteins was determined by the pepsin-pancreatin digestion method of Akeson and Stahmann (1964), as modified by Stahmann and Woldegiorgis (1975).
Reagents

Pepsin solution: 10 mg of pepsin was placed in a 100 ml volumetric flask and brought to volume with 0.1 N HCl.

Pancreatin solution: 26.67 mg of pancreatin was placed in a 50 ml volumetric flask and brought to volume with pH 8.0 phosphate buffer.

pH 8.0 sodium phosphate buffer (0.1M): 21.92 g of $K_2HPO_4 \cdot 3H_2O$ and 0.523 g of anhydrous $KH_2PO_4$ were dissolved in water and diluted to one liter.

14% sulfosalicylic acid solution: 14.29 g of sulfosalicylic acid was dissolved in distilled water and diluted to 100 ml. The solution was filtered through Whatman No. 1 into a dark bottle.

Citrate sample diluter pH 2.2 (See DNFB reagents).

50 ppm merthiolate solution.

Procedure

50 mg of protein sample was incubated with 7.5 ml of pepsin solution in a shaking water bath at 37°C for 3 hr. After neutralization with 3.75 ml of 0.2 N NaOH, 3.75 ml of pancreatin solution and 0.25 ml of 50 ppm merthiolate was added. The digestion mixture was incubated for an additional 24 hrs in a shaking water bath at 37°C.

To precipitate the undigested protein and peptides, 5.0 ml of digestion mixture was mixed with 5.0 ml of 14%
sulfosalicylic acid solution, to bring the final concentration of sulfosalicylic acid to 4.7%. The flasks were shaken for 30 minutes and the pH was adjusted to 2.2 by adding drops of 6 N NaOH.

The digests were then transferred to a 15 ml graduated tube and brought to volume with pH 2.2 citrate buffer. The solution was centrifuged at 1,000 x g for 30 minutes, filtered through Whatman No.42 and stored in the freezer at -20°C for amino acid analysis.

The same procedure was carried out for an enzyme blank that was prepared by incubation with the protein sample omitted.

Calculation:

15.25 ml of digestion mixture (final volume) contained 50 mg protein; thus, 5 ml of digestion mixture contained 16.39 mg protein. 5.00 ml of digestion mixture mixed with sulfosalicylic acid and made up to 15 ml with citrate buffer, contained 16.39 mg protein; 0.5 ml used for amino acid analysis, contained 0.546 mg protein.

The areas under the peaks of the effluent curve were calculated by multiplying the height of the peak by the width at half the height, and dividing by the constant for the given amino acid. Micromoles, or micrograms of amino acid per volume applied, were obtained.
If casein had 13.899 μg lysine/0.5 ml:

\[
\frac{13.899 \, \mu g \, lysine/0.5 \, ml}{0.546 \, mg \, protein/0.5 \, ml} \times \frac{1 \, mg}{1000 \, \mu g} \times 100 \, mg =
\]

2.544 mg lysine/100 mg casein.
RESULTS AND DISCUSSION

The protein content of the samples, as determined by the rapid micro-Kjeldahl method of Concon and Soltess (1973) is shown in Table 3.

A. The dinitrobenzene sulfoic acid (DNBS) method

The method described by Concon (1975a) employs the reagent sodium dinitrobenzenesulfonate (DNBS) in a urea-phosphate-phenylmercuric chloride solution, to complex the ε-amino groups of lysine. After colour development, the interferences are eliminated by ether extraction. The optical density is then measured at 385 nm and the amount of available lysine is calculated from a standard curve. Table 4 shows the available lysine content of protein samples as determined by the DNBS method.

From the samples subjected to the DNBS procedure, only 1.5 N HCL gluten and whole egg solids had a high blank value. This is often encountered in undefatted materials or in those cereal grains with highly pigmented outer layers. According to Concon (1975b), for the DNBS method, lipids need not be removed except in heavily pigmented varieties of barley, oats, rye, and wheat. However, subjecting the gluten and egg samples to fat extraction might have produced lower blank values.

The sample blanks for β-lactoglobulin, lysozyme, casein and RNase-S-peptide gave lower absorbance readings
TABLE 3. Protein content of samples assayed for lysine availability

<table>
<thead>
<tr>
<th>Sample</th>
<th>g protein/100 g sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 N HCl Gluten</td>
<td>85.20</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>63.43</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>89.20</td>
</tr>
<tr>
<td>Casein</td>
<td>72.30</td>
</tr>
<tr>
<td>Whole Egg (freeze-dried)</td>
<td>37.26</td>
</tr>
</tbody>
</table>
TABLE 4. Available lysine content of protein samples as determined by the dinitrobenzene sulfonic acid (DNBS) method. (Values in g lysine/100 g protein).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Experiment No.</th>
<th></th>
<th>Ave</th>
<th>Reported values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>Ave</td>
</tr>
<tr>
<td>Gluten</td>
<td>1.801</td>
<td>1.668</td>
<td>1.712</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.818</td>
<td>1.668</td>
<td>1.715</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.838</td>
<td>1.702</td>
<td>1.764</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>1.819</td>
<td>1.679</td>
<td>1.730</td>
<td>1.748</td>
</tr>
<tr>
<td>Casein</td>
<td>7.413</td>
<td>7.575</td>
<td>7.550</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.408</td>
<td>7.504</td>
<td>7.501</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.496</td>
<td>7.575</td>
<td>7.618</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>7.439</td>
<td>7.551</td>
<td>7.556</td>
<td>7.515</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>5.463</td>
<td>5.327</td>
<td>5.507</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.545</td>
<td>5.396</td>
<td>5.402</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.414</td>
<td>5.430</td>
<td>5.402</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>5.474</td>
<td>5.384</td>
<td>5.437</td>
<td>5.432</td>
</tr>
<tr>
<td>β-lactog.</td>
<td>12.354</td>
<td>11.837</td>
<td>12.723</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12.428</td>
<td>11.837</td>
<td>12.797</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12.283</td>
<td>11.543</td>
<td>12.503</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>12.355</td>
<td>11.739</td>
<td>12.674</td>
<td>12.256</td>
</tr>
<tr>
<td>Egg</td>
<td>5.490</td>
<td>5.368</td>
<td>5.610</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.523</td>
<td>5.340</td>
<td>5.622</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.346</td>
<td>5.327</td>
<td>5.722</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>5.453</td>
<td>5.345</td>
<td>5.651</td>
<td>5.483</td>
</tr>
<tr>
<td>RNase-S-</td>
<td>10.670</td>
<td>10.618</td>
<td>10.644</td>
<td></td>
</tr>
<tr>
<td>peptide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Available lysine content, based on known amino acid sequence, from Purcell et al., 1976.
than the reagent blank. In this case, the negative value was ignored; obviously, no absorbance is contributed by the sample without dinitrophenylation. The higher absorbance of the reagent blank may come from the reaction of DNBS with impurities in the reagents or simply contaminations (Concon, 1971).

The time of dinitrophenylation greatly affects the precision of the DNBS results since the absorbance of the solution during color development increases with time. A change of about 0.006 absorbance units per minute per ml with a lysine concentration of 0.4 moles per ml at pH 12 and 40°C, can be expected (Concon, 1971). In this thesis, about 8 minutes elapsed between addition of DNBS to the first tube and addition of the reagent to the last tube, before incubation at 40°C. Thus, an absorbance error of 0.048 units or less, since at room temperature very little dinitrophenylation occurs, can be expected in the results.

Studying the reactivity of TNBS with amino acids and peptides, Freedman and Radda (1968) showed that, at room temperature, pH 7.4, the ε-amino groups of lysines were 30 times more reactive to TNBS than were the α-amino groups. When the neighboring carboxyl group was amidated or joined in a peptide bond, the α-amino groups became even less reactive. These authors concluded that the decreased nucleophilicity of the amino group as a result of amidation
or peptide bond formation of the carboxyl radical, accounted for the decreased reactivity of TNBS. It was then suggested that DNBS, a reagent less electrophilic than TNBS, might be more specific for the ε-amino group. The absence of one extra ortho nitro group in DNBS is expected to diminish the corresponding electrophilicity of the sulfonate radical. Steric and other electrostatic factors may also influence the reactivity of both DNBS and primary amino groups. Therefore, under the reaction conditions used, a virtual disappearance of the absorbance, due to α-amino groups may result. However, using model lysine derivatives with different numbers of α- and ε-amino groups, it has been found that DNBS also reacts with the α-amino groups to a certain extent. Table 5 indicates the results of this experiment. ε-formyl-lysine which has one free α-amino group, when reacted with DNBS, shows a 385 nm absorbance value of 0.091, but α-formyl-lysine that has one free ε-amino group, shows an absorbance value of 0.289. The expected values of lysine, lysyl-lysine, lysyl-alanine and ribonuclease-S-peptide, have been calculated assuming DNBS specificity for the ε-amino group. The difference between the experimental and the expected values, shows the extent to which the α-amino group reacts with DNBS. The difference is 0.095 for lysine, 0.069 for the dipeptide lysyl-lysine, and only 0.026 for RNase-S-peptide. A difference of 0.001 was
<table>
<thead>
<tr>
<th>Amino acid or peptide (1)</th>
<th>Number of $\varepsilon$-NH$_2$ $\alpha$-NH$_2$</th>
<th>Experimental</th>
<th>Expected (2)</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\varepsilon$-formyl-lysine</td>
<td>0 1</td>
<td>0.091</td>
<td>0.000</td>
<td>0.091</td>
</tr>
<tr>
<td>$\alpha$-formyl-lysine</td>
<td>1 0</td>
<td>0.289</td>
<td>0.289</td>
<td>0.000</td>
</tr>
<tr>
<td>Lysine</td>
<td>1 1</td>
<td>0.384</td>
<td>0.289</td>
<td>0.095</td>
</tr>
<tr>
<td>Lysyl-lysine</td>
<td>2 1</td>
<td>0.647</td>
<td>0.578</td>
<td>0.069</td>
</tr>
<tr>
<td>Alanine</td>
<td>0 1</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Lysyl-alanine</td>
<td>1 1</td>
<td>0.290</td>
<td>0.289</td>
<td>0.001</td>
</tr>
<tr>
<td>RNase-S-peptide</td>
<td>2 1</td>
<td>0.604</td>
<td>0.578</td>
<td>0.026</td>
</tr>
</tbody>
</table>

(1) 2.5 mM solutions.

(2) If DNBS is specific for $\varepsilon$-NH$_2$
found for the dipeptide lysyl-alanine, indicating that the 
α-amino group of alanine is not measured by the DNBS 
technique.

From the above figures it can be seen that when 
lysine is at the N-terminal position in a protein, the re-
sult will be slightly higher than the true content of 
available lysine, since the α-amino group is contributing to 
the results.

RNase-S-peptide is a 20-residue component which has 
two lysine units, one of them located at the N-terminal 
position (Richards and Vithayathil, 1959). In this peptide, 
the ratio of ε- to α-amino groups is 2 to 1, and thus, the 
contribution of the α-amino group to the result is compara-
tively high (0.026). If the expected value for RNase-S-
peptide, that is 0.578, is used to calculate the available 
lysine content in g per 100 g of protein, the result is 10.2. 
The same figure was calculated by Purcell et al. (1976), 
based on known amino acid sequence.

In this work, the available lysine content of RNase-
S-peptide calculated from an absorbance value of 0.604, was 
found to be 10.64 g per 100 g of protein. Purcell et al. 
using a reagent less specific than DNBS, such as fluorescamine, 
which labels the ε- as well as the α-amino groups of proteins, 
obtained a value of 15.3 g lysine per 100 g of RNase-S-peptide.

In proteins and higher polypeptides that have N-
terminal lysine, the ratio would be that of several ε-amino
groups to one α-amino group. In this case, the contribution of the α-amino group to the results, becomes negligible. Thus, the lysine availability as determined by the DNBS method, will give reliable data for proteins containing a substantial number of ε-amino groups per chain.

α- and ε-carbobenzoxy-lysines were also used for the DNBS specificity study. These derivatives did not show any absorbance value when reacted with DNBS. This behavior could be explained in terms of a steric benzy-benzyl hindrance.

B. The trinitrobenzene sulfonic acid method (TNBS)

The reagent 2,4,6-trinitrobenzene sulfonic acid was used to complex with the free ε-amino group of lysine, and the resulting ε-TNP-lysine was determined spectrophotometrically after 90 min of acid hydrolysis.

This method gave values for available lysine, in fair agreement with those reported in the literature, as shown in Table 6.

TNBS has been found to react with various interfering compounds; thus, the reported values are sometimes contradictory. Holsinger et al. (1970) and Holsinger and Posati (1975) reported anomalously high results when the TNBS procedure of Kakade and Liener (1969) was applied to caseins. The available lysine content for k-casein was 15% higher than the total lysine content, and whole casein values were 10-15% high. They ascribed this finding to the presence of d-glucosamine and d-galactosamine, as well as to other
TABLE 6. Available lysine content of protein samples as determined by the trinitrobenzene sulfonic acid (TNBS) method. (Values in g lysine/100 g protein).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Experiment No.</th>
<th>Reported values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Gluten</td>
<td>1.388</td>
<td>1.370</td>
</tr>
<tr>
<td>Average</td>
<td>1.408</td>
<td>1.373</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>5.734</td>
<td>5.584</td>
</tr>
<tr>
<td>Average</td>
<td>5.796</td>
<td>5.594</td>
</tr>
<tr>
<td>Casein</td>
<td>7.594</td>
<td>6.913</td>
</tr>
<tr>
<td>Average</td>
<td>7.607</td>
<td>6.874</td>
</tr>
<tr>
<td>Average</td>
<td>13.002</td>
<td>14.228</td>
</tr>
<tr>
<td>Egg</td>
<td>6.134</td>
<td>7.028</td>
</tr>
<tr>
<td>Average</td>
<td>6.163</td>
<td>7.141</td>
</tr>
</tbody>
</table>

Available lysine as determined by the TNBS method:
1. From Finley and Friedman, 1973.
2. From Kakade and Liener, 1969.
3. From Holsinger et al., 1970.
5. From Hall et al., 1973.
uncharacterized glycoprotein fragments in the casein micelle. Amino sugars react with TNBS to form colored derivatives which are not removed by ether extraction. αs- and β-caseins known to be carbohydrate-free, gave available lysine contents, either equal to or less than the total lysine present.

On the other hand, low TNBS values have been reported by Posati and her associates (1975) in products containing lactose. These authors found that the presence of lactose in levels approaching the lysine:lactose ratio in dehydrated whey powders, resulted in severe destruction of the ε-TNP-lysine derivative.

Hall et al. (1975) and Posati et al. (1972) have stressed the importance of a small sample size in order to obtain a reliable data with TNBS. A sample of 0.05 mg of bovine serum albumin gave about 89% available lysine, whereas a sample size of 0.7 mg gave only 75% available lysine (Posati et al., 1972). On the other hand, Eklund (1976) obtained reproducible results by employing a 30 mg sample.

There is also some conflict in the literature regarding the reactivity of free lysine towards TNBS. Lysine reacts with TNBS to form initially α,ε-diTNP-lysine, which Kotaki and Satake (1964) showed to be unstable in hot 6 N hydrochloric acid; 81% ε-TNP-lysine and 17% free lysine was recovered after acid hydrolysis of α,ε-diTNP-lysine (Okuyama and Satake, 1960). Ousterhout and Wood (1970) found, however, that free lysine was directly converted to ε-TNP-
lysine. Hall and associates (1973) found that recoveries of free lysine and ε-TNP-lysine added as internal standards to protein suspensions which were then subjected to the TNBS reaction and acid hydrolysis were variable. Furthermore, since α,ε-diTNP-lysine is ether soluble, in proteins where lysine is at the N-terminal position, this derivative or small peptides containing it, would be extracted into the ether phase and would escape detection.

Hall et al. (1975) found variable results for available lysine in carbohydrate-rich materials. According to them, browning and charring produced during acid hydrolysis accounted for the low results for available lysine. ε-TNP-lysine was adsorbed on to the carbon particles (from charring) which were then removed during the ether extraction process. When the sample size was decreased, Hall et al. found that the available lysine measured by TNBS reaction exceeded the value for the total lysine. In materials rich in starches, the purified starches themselves reacted with TNBS to give an apparent lysine content. From these findings, Hall et al. recommended that the TNBS reaction be carried out at 30°C for 30 minutes, for determining available lysine, in feeds and cereals rich in carbohydrates.

Not only carbohydrates and sugars interfere in the determination of lysine availability with TNBS, but naturally occurring amines such as agmatine, spermine, spermidine and taurine, react with TNBS and can be measured as available
lysine. Cadaverine, hydroxylysine, ornithine and putrescine, if present in samples of tissues that have undergone some decomposition, can give misleadingly high results.

There is some disagreement in regard to the standard used to draw the standard curve for the TNBS method. Kakade and Liener (1969) and Eklund (1976) used ε-TNP-lysine as a standard. Graded levels of ε-TNP-lysine were subjected to the same procedure as the protein samples. Since ε-TNP-lysine was not available in the U.K., Hall et al. (1973) proposed the use of lysine as a standard. They demonstrated that the product from pure DL-lysine after reaction with TNBS and ether extraction had virtually the same absorbance value per unit mass as a solution of synthetic ε-TNP-lysine. However, when lysine was reacted with TNBS and subjected to acid hydrolysis and ether extraction, we found a molar absorptivity of $1.04 \times 10^4$ (moles/liter)$^{-1}$ cm$^{-1}$. ε-TNP-lysine, subjected to the same procedure, had a molar absorptivity of $0.89 \times 10^4$ (moles/liter)$^{-1}$ cm$^{-1}$. But, when ε-TNP-lysine was read directly at 346 nm, the molar absorptivity was found to be $1.46 \times 10^4$ (moles/liter)$^{-1}$ cm$^{-1}$ which is in agreement with the value reported by Kakade and Liener (1969).

In the present work, TNBS-available lysine was determined from a standard curve which was built by reading directly the 346 nm absorbance of graded levels of ε-TNP-lysine. According to Hall et al. (1973) about 100% of ε-TNP-lysine can be recovered from either a) ε-TNP-lysine directly in 1 N HCl; b) ε-TNP-lysine in 1 N HCl after ether
extraction, or c) ε-TNP-lysine ether-extracted after 2 hr in approximately 7 N HCl at 100°C.

From the results presented above, it can be seen that many compounds interfere with the TNBS-available lysine determination. The method would require several corrections and modifications before it can yield reliable data.

C. The fluorodinitrobenzene (FDNB) method.

The FDNB difference method utilizes the reagent 1-fluoro-2,4-dinitrobenzene (FDNB) to bind the free ε-amino group of lysine. An ε-DNP-lysine complex is formed, which resists acid hydrolysis. When the FDNB-reacted sample is hydrolyzed and analyzed for lysine by the method of Moore et al. (1959), the lysine which was bound and not available for combination with FDNB, is measured. This is considered to be unavailable lysine. Available lysine, which was bound by FDNB, is calculated as the difference between total and unavailable lysines.

Table 7 shows the lysine found after acid hydrolysis, the unavailable lysine as determined by the FDNB procedure, and the available lysine content of the protein samples. The results are in good agreement with the reported values. In the case of β-lactoglobulin, the available lysine content was found to be 11.27%. Purcell et al. (1976) reported a value of 10.5% for available lysine in β-lactoglobulin, based on
TABLE 7. Available lysine content of protein samples as determined by the fluoro dinitrobenzene (FDNB) difference method.

<table>
<thead>
<tr>
<th>Protein</th>
<th>TOTAL LYSINE</th>
<th></th>
<th>UNAVAILABLE LYSINE</th>
<th>AVALI-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment No.</td>
<td>Reported Value</td>
<td>Experiment No.</td>
<td>LABLE</td>
</tr>
<tr>
<td></td>
<td>1  2  3  4  AVE</td>
<td></td>
<td>1  2  3  AVE</td>
<td></td>
</tr>
<tr>
<td>Gluten</td>
<td>1.67  1.69 1.73 1.71 1.70</td>
<td>1.251</td>
<td>0.17  0.13   - 0.15</td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>7.42  7.83 7.59 7.68 7.63</td>
<td>6.12/3</td>
<td>0.22  0.25  0.22 0.23</td>
<td></td>
</tr>
<tr>
<td>Lysozyme</td>
<td>5.28  5.36 5.35  - 5.33</td>
<td>5.75/1</td>
<td>0.19  0.17   - 0.18</td>
<td></td>
</tr>
<tr>
<td>β-lactog.</td>
<td>13.60 12.29 13.10 14.41 13.35</td>
<td>11.25/5</td>
<td>2.20  1.93  2.11 2.08</td>
<td></td>
</tr>
<tr>
<td>Egg</td>
<td>6.92  6.83 7.15 6.46 6.84</td>
<td>6.24/6</td>
<td>0.25  0.30  0.32 0.29</td>
<td></td>
</tr>
</tbody>
</table>

The values are given in g lys/100 g protein.

2. Total and unavailable lysine determined by the FDNB Difference procedure, from Booth, 1971.


4. Total lysine as determined by acid hydrolysis, from Stahmann and Woldegiorgis, 1975.

5. Total lysine by composition, from Tristram, 1953.

6. Total lysine from compositional data, from Jenness and Patton, 1959.

7. Total lysine from compositional data, from Orr and Watt, 1957.


12. Available lysine calculated from known amino acid sequence, from Purcell et al., 1976.
known amino acid sequence. Porter (1948) have reported that some proteins such as β-lactoglobulin and serum globulins, include lysine groups that react with FDNB only after denaturation, which may be effected with ethanol, guanidine or mild acid. It might be advisable to test the effect of prior denaturation on β-lactoglobulin, to see whether a higher FDNB-reactive lysine result is obtained.

The original FDNB procedure (Carpenter, 1960) is known to yield poor recoveries of ε-DNP-lysine and variable results, especially when applied to cereals and oilseed meals. The poor performance of the method is associated with the destruction of ε-DNP-lysine by carbohydrates during hydrolysis, and the formation of other yellow products which are not easily separated from ε-DNP-lysine (Carpenter et al., 1957; Rao et al., 1963). Partial destruction of ε-DNP-lysine does not cause difficulties when available lysine is determined by the FDNB difference procedure, since this derivative is not measured with this technique; ε-DNP-lysine is mathematically calculated by difference.

The difference technique suffers from a major disadvantage, that it is extremely time consuming; 18 hr (unavailable lysine) and 24 hr (total) of acid hydrolysis are required for each determination of lysine availability. Subsequent removal of HCl from the sample and reduction of the
sample to dryness on a rotary evaporator, is, however, the most time consuming step. Hubbard and Finney (1976) have described a technique involving an Evapo-Mix evaporator, which greatly reduces the time and attention otherwise required. With this system, 10 samples of 5.5 ml of solution were reduced to dryness in about 35 minutes.

Although time consuming, the FDNB difference technique is the most accurate means presently available for the chemical determination of available lysine. The values obtained will include free lysine and N-terminal lysine.

D. Pepsin-pancreatin digestion test.

Although in the final analysis, the nutritional quality of a protein must be assessed by feeding trials, the enzymatic methods for protein quality evaluation are very useful for preliminary quality evaluation. In vitro enzymatic digestion methods attempt to imitate the action of the mammalian digestive system. Compared to animal assays, the enzymatic methods have several advantages. They are less expensive and require less time than bioassays; they show less variation than protein efficiency ratio assays with rats; a single assay can indicate the relative amounts of essential amino acids contained in and released by the enzyme from the protein under assay.

The enzymic methods have been used to monitor the adverse effects of processing operations on proteins, that
may decrease the digestibility of the protein, reduce the availability of lysine or oxidise sulfur amino acids. Stahmann (1977) found that peroxidase or polyphenoloxidase in the presence of chlorogenic acid and protein, upon acid hydrolysis, revealed no destruction with increasing concentrations of chlorogenic acid and oxidase enzymes, but enzymatic hydrolysis revealed a complete destruction of methionine.

Even though the enzymic in vitro digestion is not complete, so that the values obtained are only relative, the amount of the limiting amino acid released by enzymic hydrolysis is a good indicator of amino acid availability. Lysine that has its ε-amino group blocked has no nutritive value since those groups are not susceptible to enzymatic hydrolysis and the lysine could not be freed.

The original method of Akeson and Stahmann (1946) used picric acid as a precipitant of undigested protein. However, this procedure was found to be very inconvenient since it was necessary to pass the treated sample through a column in order to eliminate the picric acid. In 1975, the author suggested the use of sulfosalicylic acid to precipitate the undigested protein. This is an advantageous modification since this reagent is colorless, ninhydrin negative and can be directly applied to the columns for amino acid analysis.
Menden and Cremer (1966) claimed that it is preferable to digest the protein with a high concentration of enzyme for a short period, so that autohydrolysis of enzymes becomes negligible. The pepsin-pancreatin digestion test of Stahmann and Woldegiorgis (1975) uses a digestion time of 27 hours, and an enzyme blank must be run along with each experiment. This blank value ranged between 0.585 and 1.426 μg of lysine per ml that was substracted from the sample value.

Table 8 summarizes the results for available lysine as determined by the pepsin-pancreatin digestion test. The results indicate the relative amount of lysine released by the enzymes under the conditions specified for the test.

A correlation coefficient of 0.995 was obtained between the enzymatic test and the available lysine determined by the FDNB official method. This result is in close agreement with the finding of Stahmann and Woldegiorgis (1975) who reported a correlation coefficient of 0.9974 between the available lysine determined by the modified Carpenter method (Booth, 1971) and the lysine released by the digestive enzymes. Bujard et al. (1967) also reported a high correlation for the available lysine as determined by the pepsin-pancreatin method of Mauron et al. (1955), by in vivo feeding and by the

(Hamilton, 1962).
### TABLE 8. Amount of lysine released from protein samples subjected to pepsin-pancreatin digestion.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Experiment No.</th>
<th>Ave Reported values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Casein</td>
<td>3.463</td>
<td>4.181</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>5.374</td>
<td>5.690</td>
</tr>
<tr>
<td>1.5 N HCl Gluten</td>
<td>0.542</td>
<td>0.589</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>2.200</td>
<td>2.357</td>
</tr>
<tr>
<td>Egg</td>
<td>2.703</td>
<td>2.762</td>
</tr>
</tbody>
</table>

The results are given in g of lysine per 100 g protein.


Carpenter method, for a series of heat treated milk samples. Stahmann and Woldegiorgis (1975) demonstrated that only the enzymatic method was able to show an increase in available lysine following steaming of soybean meal. It is well known that raw soybeans contain a trypsin inhibitor, and the increase in the liberation of methionine, lysine, arginine and histidine confirms that steaming destroys the inhibitor.

It is apparent that the chemical methods for protein quality may not reveal the availability of lysine when the proteins tested contain an inhibitor of one of the proteolytic enzymes that act during digestion.

Sometimes, it might be difficult to correlate chemical and biological availability since the latter could also involve protein digestibility which may not be related to the reactivity of the ε-amino group of lysine.
GENERAL DISCUSSION

A summary of the results for available lysine as determined by the FDNB difference procedure, the DNBS and TNBS methods, and the pepsin pancreatin digestion test is shown in Table 9. For comparison of the methods, the regression lines and the correlation coefficients were estimated according to the procedure described by Deming (Wakkers et al., 1975). The "classical procedure" to estimate the regression line, assumes that one of the methods, used as the reference, is not subject to error. However, this is not always true. Deming's procedure has the advantage that estimates are obtained for the random errors of both methods.

Good agreement was obtained between the FDNB official method and the DNBS technique, with a correlation coefficient of 0.989 (Figure 12). When the TNBS method was compared to the FDNB difference technique, a correlation coefficient of 0.988 was found (Figure 13).

The pepsin pancreatin test indicated the relative amount of lysine released by the enzymes under the conditions specified for the test. A correlation coefficient of 0.995 was found between the FDNB method and the enzymic test (Figure 14).

Figures 15, 16 and 17 show the regression lines for comparison between the enzymatic test and the DNBS method \((r = 0.998)\), the enzymatic test and the TNBS method \((r = 0.987)\)
TABLE 9. Lysine availability determined by the FDNB official procedure, the DNBS and the TNBS methods, and the enzymatic test.

<table>
<thead>
<tr>
<th>Sample</th>
<th>FDNB</th>
<th>DNBS</th>
<th>TNBS</th>
<th>Enzymatic Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluten</td>
<td>1.55</td>
<td>1.75</td>
<td>1.46</td>
<td>0.56</td>
</tr>
<tr>
<td>Casein</td>
<td>7.35</td>
<td>7.52</td>
<td>7.14</td>
<td>3.8</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>5.15</td>
<td>5.43</td>
<td>5.73</td>
<td>2.31</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>11.27</td>
<td>12.26</td>
<td>13.93</td>
<td>5.69</td>
</tr>
<tr>
<td>Whole egg</td>
<td>6.55</td>
<td>5.48</td>
<td>6.71</td>
<td>2.77</td>
</tr>
</tbody>
</table>

The values are given in g per 100 g of protein.
FIGURE 12. Comparison between the FDNB difference technique and the DNBS method. The % of lysine is determined by g lysine/100 g protein.
Comparison between the FDNB difference technique and the TNBS method. The % of lysine is determined by $g$ lysine/100 $g$ protein.

$r = 0.988$

- gluten
- lysozyme
- egg
- casein
- β-lactoglobulin
FIGURE 14. Comparison between the FDNB difference method and the enzymatic digestion test. The % of lysine is determined by g lysine/100 g protein.
FIGURE 15. Comparison between the enzymatic digestion test and the DNBS method. The % of lysine is determined by g lysine/100 g protein.
FIGURE 16. Comparison between the enzymatic digestion test and the TNBS method. The % of lysine is determined by g lysine/100 g protein.

$\text{ENZ. TEST (\% lysine)}$

$\text{TNBS (\% lysine)}$

$r = 0.987$

- gluten
- lysozyme
- egg
- casein
- $\beta$-lactoglobulin
FIGURE 17. Comparison between the TNBS and the DNBS methods. The % of lysine is determined by g lysine/100 g protein.

\[ r = 0.995 \]

- gluten
- lysozyme
- egg
- casein
- \( \beta \)-lactoglobulin
and the TNBS and DNBS method \( (r = 0.995) \), respectively.

The standard deviation of the random error for each method is shown in Table 10. A multiple range test (Duncan's test) showed that the TNBS method produced significantly lower repeatability as compared to the other three methods. No significant difference in precision was found among the FDNB official technique, the DNBS method and the enzymatic test.
TABLE 10. Standard deviation of the random error (in g lysine/100 g protein) in the available lysine methods, as estimated by Deming's procedure.

<table>
<thead>
<tr>
<th>Methods compared</th>
<th>FDNB</th>
<th>DNBS</th>
<th>TNBS</th>
<th>Enzymatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDNB and DNBS</td>
<td>0.577</td>
<td>0.546</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FDNB and TNBS</td>
<td>0.366</td>
<td></td>
<td>0.663</td>
<td></td>
</tr>
<tr>
<td>FDNB and Enz.</td>
<td>0.419</td>
<td></td>
<td></td>
<td>0.187</td>
</tr>
<tr>
<td>TNBS and DNBS</td>
<td></td>
<td>0.363</td>
<td>0.672</td>
<td></td>
</tr>
<tr>
<td>Enz. and DNBS</td>
<td></td>
<td>0.256</td>
<td></td>
<td>0.308</td>
</tr>
<tr>
<td>Enz. and TNBS</td>
<td></td>
<td></td>
<td>0.683</td>
<td>0.399</td>
</tr>
</tbody>
</table>
CONCLUSION

From the results presented in this thesis it can be seen that lysine availability in casein, acid solubilized gluten, egg, \( \beta \)-lactoglobulin and lysozyme, as determined by the official FDNB difference technique gave results in close agreement with the reported values. Although the difference technique is extremely time consuming, it is probably the most accurate means presently available for determination of lysine availability. It is not affected by the destruction of \( \varepsilon \)-DNP-lysine during acid hydrolysis.

The lysine released by the pepsin-pancreatin digestion correlated well with the available lysine as determined with the FDNB chemical method.

The DNBS and TNBS methods gave results in close agreement with the results obtained with the FDNB official technique. However, the data from the TNBS method showed a wide range of variability and significantly lower repeatability.

The use of lysine derivatives to study the specificity of DNBS for the \( \varepsilon \)-amino group of lysine, showed that DNBS reacts with the \( \alpha \)-amino group of lysine, to a certain extent. However, for proteins and higher polypeptides the contribution of the \( \alpha \)-amino group to the results becomes negligible.
Only for small peptides having N-terminal lysine will the result be slightly higher than the true content of available lysine.

The DNBS method was found to be the simplest and most reliable method for determination of available lysine, for the following reasons:

1) It does not require acid hydrolysis of the protein.
2) It is specific for the ε-amino group of lysine and practically free from interferences.
3) A large number of samples can be analyzed simultaneously in a few hours.
4) It does not require expensive and lengthy chromatographic amino acid analysis.
REFERENCES CITED

A pepsin pancreatin digest index of protein 

BARAUD, J. 1957. 
Iodometric analysis of free amino nitrogen in 
protein by means of their dithiocarbamate 

Determination of the nutritive value of pro­
teins by chemical analysis. IN: Progress in 
Meeting Protein Needs in Infants and Preschool 
Children, p. 407, Publ. 843, Natl. Acad. Sci., 

New methods of assessing protein quality. 

Biological evaluation of proteins: A new aspect. 

Determination of the available lysine in foods. 

Measurement of available lysine in heated and 
unheated food stuffs by chemical and biological 

BOOTH, V.H. 1971.

Influence of amino acid level in the diet upon amino acid oxidation by the rat. J. Nutr. 102:27.


Interference by cyanide with the measurement of papain hydrolysis. J. Sci. Food Agric. 20:364.

The availability of lysine in wheat, flour, bread

CARPENTER, K.J. 1960.
The estimation of the availability of lysine in

CARPENTER, K.J. and A.M. Ellinger. 1955a.
The estimation of available lysine in protein con­

CARPENTER, K.J. and A.M. Ellinger. 1955b.
Protein quality and available lysine in animal

CARPENTER, K.J., A.M. Ellinger, M.I. Munn and E.J. Rolfe.
1957.
Fish products as protein supplements to cereals.

1963.
Growth assay with chicks for the lysine content

CARPENTER, K.J. and D.S. Miller. 1963.
IN: Evaluation of Protein Quality, Report of an
International Committee on Protein Malnutrition,
Food and Nutrition Board, National Academy of
Sciences - National Research Council, Publication 1100, p.7. National Academy of Sciences,
Washington, D.C.
CARPENTER, K.J. and J. Bjarnason. 1969.


New amino acids derived from reactions of ε-amino groups in proteins with α,β-unsaturated compounds. Biochem. 6: 3766.


CONCON, J.M. 1975a.

CONCON, J.M. 1975b.
 Rapid micro Kjeldahl digestion of cereal grains
 53: 35.

CONKERTON, E.J. and V.L. Frampton. 1959.
 Reaction of gossypol with free ε-amino groups
 81: 130.

COUCH, J.R. 1975.
 Collaborative study on the determination of
 available lysine in proteins and feeds.
 J. AOAC. 58: 599.

 A comparison of chemical methods for the deter­
 mination of available lysine in various proteins.

 Effects of severe alkali treatment of proteins
 in amino acid composition and nutritive value.
 J. Nutr. 98: 45.

DVORAK, D. 1968.
 Availability of essential amino acids from
 19: 71.

 The reaction of 2,4-dinitrobenzene sulfonic
 acid with free amino groups of proteins.
 J. Amer. Chem. Soc. 75: 4583.
EKLUND, A. 1976.
On the determination of available lysine in casein and rapeseed protein concentrates using 2,4,6-trinitrobenzene sulfonic acid as a reagent for free epsilon amino groups of lysine. Anal. Biochem. 70: 434.

EL-NOCKRASHY, A.S 1965.
Determination of available lysine in cottonseed meal. Stärke 17: 89.


EVANS, R.J. and H.A. Butts. 1948.


FORD, J.E. 1960.

FORD, J.E. 1962.

FORD, J.E. 1965.

FORD, J.E. and D.N. Salter. 1966.


GOLDFARB, A. 1966.
A kinetic study of the reaction of amino acids and peptides with trinitrobenzene sulfonic acid. Biochem. 5: 2470.

GREENLEE, L. and P. Handler. 1964.
Xantine oxidase. VI. Influence of pH on substrate specificity, VII. Inhibition by amino group reagents. J. Biol. Chem. 239: 1096.


Observation on the use of 2,4,6-trinitrobenzene sulfonic acid for the determination of available lysine in animal protein concentrates. Analyst 98: 673.

HALL, R.J., N. Trinder and M.R. Wood. 1975.
The determination of available lysine in carbohydrate rich materials. Analyst 100: 68.

HAMLETTON, P.P.B. 1962.

The reduction of aromatic nitro groups during acid hydrolysis in the presence of carbohydrates and its bearing on the colorimetric determination of ε-dinitrophenyl-lysine. Biochem. J. 76: 54 P.

ε-(γ-glutamyl) lysine cross linkage in citrulline containing protein fractions from hair. Biochem. 10: 624.

Modification of amino groups in inhibitors of proteolytic enzymes. Biochem. 6: 541.

Efficiency of protein utilization in young rats at various levels of intake. J. Nutr. 100: 1173.


Anomalous results obtained in the determination of available lysine in casein using 2,4,6-trinitrobenzene sulfonic acid. J. Dairy Sci. 53: 1638.


Turnip peroxidase. V. The effects of several chemical reagents upon the reactivity of turnip peroxidase. J. Biochem. 48: 375.

The nutritive value of bread flour proteins as affected by practical supplementation with lactalbumin, non fat milk solids, soybean proteins, wheat gluten and lysine. J. Nutr. 64: 151.


IKENAKA, T. 1959.
Chemical modification of Taka-amylase A.
I. Dinitrophenylation of Taka-amylase A.
J. Biochem. 46: 177.


Losses in available lysine during thermal processing of soy protein model systems.
J. Food Sci. 41: 816.

Determination of available lysine in proteins.

KAI, F. 1967.

Reaction between sulfhydryl compounds and 2,4,6-trinitrobenzene-1-sulfonic acid.
J. Biochem. 55: 553.


LANDERS, R.E 1975.


Digestive acceptability of proteins as measured by the initial rate of in vitro proteolysis. J. Food Sci. 38: 173.

MATHESON, N.A. 1968.

MAURON, J. 1970.
MAURON, J. 1972.


MAURON, J. and F. Mottu. 1962.


Reductive alkylation of amino groups in proteins. Biochem. 7: 2192.

MELNICK, D., B.L. Oser and S. Weiss. 1946.
Rate of enzymic digestion of proteins as a factor in nutrition. Science 103: 326.


Estimates on the availability of amino acids in soybean oil meal as determined by chick growth assay: Methodology as applied to lysine.

Determination of the available lysine in milk and processed cheese by the reactive dye Remazol Brilliant Blue R. Z. Lebensm. -Unters. Forsch. 144: 92.

On the preparation and properties of 2,4,6-trinitrophenyl-amino acids and peptides.
J. Biochem. (Tokyo) 47: 454.


Protein quality evaluation revisited. Food Tech. 32 (5): 60.


Cross-link in fibrin polymerized by factor XIII; $\epsilon$-($\gamma$-glutamyl) lysine. Science 160: 892.

PORTER, R.R. 1948.

Factors affecting the determination of available lysine in whey with 2, 4, 6-trinitrobenzene sulfonic acid. J. Dairy Sci. 55: 1660.


Protein digestion with simultaneous dialysis. Z. Lebensm. -Unters. Forsch. 133: 73.

New method for the determination of free amino groups in intact pure proteins: Relationship to available lysine. J. AOAC. 59: 1251.


Structural studies of ribonuclease. XXI. The reaction between ribonuclease and a water-soluble carbodiimide. Biochem. 5: 99.


RHINEHART, D. 1975.
A nutritional characterization of the distiller's grain protein concentrates. M.S. thesis, Univ. of Nebraska, Lincoln, NE.


ROCKLAND, L.B. and M.S. Dunn. 1949.

Tetrahymena pyriformis as a test organism for the evaluation of protein quality. Naringsfors. 17: 118.

SANGER, F. 1945.

The spectrophotometric determination of amines, amino acids and peptides with 2,4,6-trinitrobenzene-1-sulfonic acid. J. Biochem. 47: 654.


SELIM, A.S.M. 1965.

The pepsin-digest residue (PDR) amino acid index of NPU. J. Nutr. 60: 105.

SHINODA, T. 1965.

SHORROCK, C. 1972.

Measurement of the amino acid content of fish meal proteins by chick growth assay. 2. The effects of amino acid imbalances upon estimations of amino acid availability by chick growth assay. Poultry Sci. 44: 408.

SMITH, T.A. 1972.
The physiology of the polyamides and related compounds. Endeavour 31: 22.


STAHMANN, M.A. 1977.


SUGAE, K. 1960.
Importance of fructose lysins and their decomposition products furosine and pyridosine for the quality control of foods. Lebensm. Technol. 6: 66.


Cytochrome a. VIII. Reaction of cytochrome a with chemically modified cytochrome c and basic proteins. J. Biochem. 52: 28.

THOMAS, M. 1970.


TRISTRAM, G.R. 1953.


VAN SLYKE, D.D. 1911.
A method for the quantitative determination of aliphatic amino groups. J. Biol. Chem. 9: 185.

VAN SLYKE, D.D. 1912.

Utilization of native and denatured proteins by Tetrahymena piriformis W. Arch. Biochem. Biophys. 56: 222.


The determination of available lysine. 5th Colloquium on amino acid analyses, Dùmont, France. Technicon International Division, Monograph 2, p. 42.


APPENDIX

3% DNBS REAGENT

Dissolve 6 g DNBS and 6 g activated charcoal in distilled water and make the volume up to 100 ml.

Stir for 10 min and filter through Whatman # 1. Measure the refractive index of the yellow solution and dilute it to obtain a 3% DNBS solution.

\[
\begin{align*}
\text{Refractive index of DNBS} & = 1.3406 \\
\text{Refractive index of distilled water} & = 1.3340 \\
\text{Difference} & = 0.0066
\end{align*}
\]

A difference of 0.0046 corresponds to a 3% DNBS (RI = 1.3386) solution, thus:

\[
\begin{align*}
0.0046 & \quad 3\% \\
0.0066 & \quad x \\
x & = 4.3\% \text{ DNBS}
\end{align*}
\]

If 4.3% DNBS \( \quad \) 91.5 ml (vol. after filtered)

3.0% DNBS \( \quad \) x

\[x = 131.15 \text{ ml} \]

Therefore, dilute the 4.3% DNBS solution up to 131.15 ml by adding 39.65 ml distilled water.