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STUDIES ON THE AMINO ACID COMPOSITION OF SELECTED MIDDLE EASTERN FOODSTUFFS

By

JALAL JAMALIAN

A THESIS

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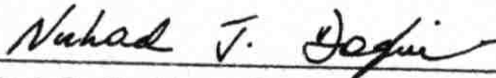

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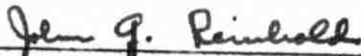

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AMINO ACID COMPOSITION

JAMALIAN

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AN ABSTRACT OF THE THESIS OF

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Title: Studies on the amino acid composition of selected Middle Eastern foodstuffs.

The world food problem is the world protein problem.

In the economically developing countries, in particular, the quantity of protein is often limited to such an extent that the quality assumes an important function. The quality of a protein depends upon its content of essential amino acids. The amino acid data available for many of the Middle Eastern foodstuffs are too few and often incomplete. The primary purpose of the present work was to complete this omission.

Amino acid data are presented for 41 items of Middle Eastern foodstuffs.

A performic acid oxidation procedure was used for the assay of sulfur amino acids, (cystine and methionine). Low methionine recoveries were observed, thus the results of preoxidation were compared with the values determined upon ordinary hydrolysis. It was concluded that the performic acid oxidation procedure was reliable for cystine and not for methionine.

Protein quality scores of the foodstuffs were calculated using FAO 1957, FAO/WHO 1965 and S/N ratio. Most of the samples analyzed were limited by sulfur amino acids, as indicated by one or more of the scoring systems. Tryptophan, lysine, and threonine were less frequently found to be limiting.

Net dietary protein calorie values of a number of mixed diets were calculated using scores obtained from the various scoring procedures. The results reflected a poor correlation between the scores. None of these scores was superior to any other in predicting the protein quality.

The fact that analytical sulfur data have been used to evaluate protein quality of diets implies that the diets are limited by sulfur amino acids and also that there is a constant ratio between the sulfur amino acid sulfur and total sulfur. This hypothesis was examined on a number of mixed diets and groups of foodstuffs; the results confirmed the use of the analytical sulfur values for

prediction of protein quality of diets and not for individual foodstuffs.

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I. INTRODUCTION

> "The enormous problem facing mankind in providing even enough food to maintain the world's rapidly increasing population at present levels of health and working efficiency is well known" (FAO, 1964).

The extent of undernutrition in the world was reported by the FAO Third World Food Survey (FAO, 1963b) which showed that some 10 to 15 percent of the people are chronically undernourished and up to a half suffer from hunger or malnutrition or both; in other words, no less than 300 to 500 million people are actually hungry. The hunger, however, is not present among all nations and definitely not to the same extent. In many of the economically more developed countries the diets of most of the population are sufficient both in quantity and quality. In the developing countries, and especially where population density is high there is shortage of food, particularly food of high quality, thus leading to extensive hunger and malnutrition (FAO, 1964).

Barnes (1963, p. 2) put more emphasis on the protein nutrition of the developing countries and reported that in these countries the amount of protein available "is often so limited that quality or biological value assumes an unusual importance".

The FAO Committee on Protein Requirements which met in Rome in 1955 used amino acid requirement values obtained experimentally for

mature human subjects and infants as "an indication of a desirable pattern of amino acid requirements". And thus the committee proposed a hypothetical reference protein, the essential amino acid pattern of which was used as a reference standard. The procedure for measuring (scoring) the quality of an unknown protein was to compare the level of essential amino acids in the protein in question against the level of essential amino acids in the reference protein.

Later it was found that the concept of a hypothetical reference protein was inadequate. The FAO/WHO Expert Group on Protein Requirements (FAO/WHO, 1965) reported, for example, that "when protein-containing foods are fed at the level needed to meet the total protein requirement, the overall pattern of available amino acids is more important in determining quality than simply the absolute amount of each of the essentials". Thus the group found that it was necessary to consider the level of total essential amino acids in relation to the total nitrogen and also the proportion contributed by each individual essential amino acid to the sum total of essential amino acids. Therefore, the need for a more detailed knowledge of the amino acid composition of foods was emphasized. The group defined an ideal dietary protein as "one with an amino acid pattern that will allow complete utilization of absorbed products for the synthesis and maintenance of body protein".

No single food can meet the requirements of the ideal amino acid pattern; however, it is possible to formulate mixtures of several foodstuffs which, through mutual supplementation, become similar to the ideal pattern.

A great deal of information now exists in the literature upon the amino acid composition of foods of which the compilation of Orr and Watt (1957) is the best known. A more recent publication is that of the Nutrition Division of Food and Agriculture Organization of the United Nations (FAO, 1963a). The sulfur amino acids are known to be the most limiting in human dietaries (Rose _et al_., 1954; Barnes, 1963, p. 2), thus the knowledge of their exact quantities is essential for evaluation of protein quality. Unfortunately these are among the ones most susceptible to destruction upon ordinary hydrolytic procedures, hence special attention must be given to these amino acids. Relatively few data, at least in the detail required for the FAO/WHO (1965) scoring procedure, exist upon many of the food items commonly consumed in the Middle East. Thus the primary purpose of the present work was to complete this omission.

II. REVIEW OF LITERATURE

Evolution of Automatic Amino Acid Analyzers

Separation of amino acids is no easy task due to the fact that they are very similar chemically and structurally. The first attempts made in the early years of the century to separate amino acids were based on chemical methods. Fischer's method (cited by Karrer, 1947, p. 278) involved esterification of amino acids with alcohol in the presence of hydrochloric acid; free amino acids were obtained from the ester hydrochlorides by the action of alkali. Another chemical procedure was that of Dakin (cited by Karrer, 1947, p. 278). The mono-amino-acids were extracted from hydrolyzates with butyl alcohol. The diamino-acids and dicarboxylic acids were insoluble in butyl alcohol and could thereby be conveniently separated. Special processes were needed for the separation of individual or groups of amino acids. The basic amino acids (arginine, lysine, histidine), for example, were precipitated by phospho-tungstic acid; tyrosine and cystine which dissolved in water with difficulty were usually isolated by fractional crystallization.

Fractionation of amino acids by chromatographic methods was first introduced in 1941 by Martin and Synge who used silica gel for the separation of acetylated amino acids; the silica gel functioned as an inert support for the aqueous phase, and the nonaqueous solvent

was allowed to flow through the silica gel. Consden et al. (1944) used paper as an inert phase which was an important advance and led to the development of a variety of paper chromatographic techniques (Block et al., 1958). Then Stein and Moore (1948) separated a number of amino acids (phenylalanine, leucine, isoleucine, methionine, tryptophan, and valine) on a column packed with potato starch. Concurrently, the photometric ninhydrin method for the chromatographic determination of amino acids was fully described by Moore and Stein (1948). In the late 1940's work was being carried out in the field of atomic energy in the USA to separate the mixture of fission products, which were a major problem to the analytical chemists. Thus ion exchange resins in combination with the technique of chromatographic elution came into the picture. Different resins were synthesized; one type was prepared by sulfonating a hydrocarbon polymer containing benzene rings (styrene with 10 percent divinylbenzene). Also various linkages were produced by making copolymers of various amounts of divinylbenzene with polystyrene. Dowex 50-X4, for example, was prepared from a mixture of styrene and divinylbenzene containing 4 percent of the latter (Lederer and Lederer, 1957, p. 74). Then in 1951, Moore and Stein separated amino acid mixtures by using Dowex 50-X8 on 0.9 X 100 cm and 0.9 X 15 cm columns. Elution was effected by increasing the pH from 3.4 to 11.0. The resin columns were found to possess higher resolving power than starch columns and were more convenient to operate. The analysis time was half compared to the time required when starch columns were used.

Using Dowex 50-X4 Moore and Stein (1954) were able to get a better resolving power than the previously studied Dowex 50-X8. They were thus able to separate the more complex mixtures involving 25 to 30 free amino acids and related compounds in blood plasma and tissue extracts; a considerable advance on the procedures used for separating the 18 to 20 amino acids present in protein hydrolyzates. Up until this time the eluted amino acids were collected using a fraction collector in a series of test tubes to which the ninhydrin reagent was added. These tubes were then heated in a water bath and after color development the intensity was determined by a colorimeter. Then Moore _et al_. (1958) chromatographed amino acids on sulfonated polystyrene resin particles which were graded for better resolving capacity by the modified hydraulic method of Hamilton (1958). The analytical procedure was completely automated for the first time by Spackman _et al_. (1958). This automatic technique was employed in the study to follow; hence it will be dealt with in more detail later.

Piez and Morris (1960) modified the automatic procedure of Spackman _et al_. (1958) by using a single 133 X 0.9 cm column packed with Dowex 50-X12 (25 to 32 u size) and eluting amino acids with buffer solutions of continuously changing pH (autograd system). Complete analysis of the hydrolyzate took 24 hours. This procedure was claimed to be suitable for the analysis of many biological amino acid mixtures. One main drawback to the autograd system was the continuous rise in the baseline of the peaks as they were recorded on the chart.

Further advance was made by Hamilton _et_ _al_. (1960) who studied the ion exchange chromatography of amino acids with due attention paid to the mechanism of diffusion (mass transfer).

In order to speed up the method of Spackman _et_ _al_. (1958) an eight column automatic equipment was constructed by Simmonds and Rowlands (1960, pp. 259-268). The apparatus included a unit which fractionated the effluent from each column, treated with ninhydrin, diluted and estimated the color produced. The measured absorbances were recorded on a paper tape; integration of the recorded values completed the analyses. Six complete analyses for the common amino acids were possible in 48 hours. Simultaneous analysis of different samples on different columns could also be performed.

Rinetti and Traineas (1961) used an autoanalyzer with a pH gradient from 3.0 to 5.7 flowing at the rate of 30 ml per hour. The total analysis time required was 25 hours.

Simultaneous with the developments in continuous flow, color development and photometry, improvements were being made in the fraction collector systems. For example, a photoelectric drop counting fraction collector was employed by Eastoe (1961) with a modification of Moore and Stein (1951) ion exchange method for the separation of amino acids. An increased sensitivity was effected by a reduction in the diameter of the resin column from 0.9 to 0.4 cm with a corresponding drop of the original volume of the fractions collected. The sharpness and resolution of the peaks obtained for amino acids were not affected by a decrease in the diameter of the column.

To improve the resolution of basic amino acids Kominz (1962) used a 50 cm column on an automatic amino acid analyzer at 50°C with a pH 5.28 (0.70 N) buffer and accomplished the analysis in 6 hours.

While the use of ninhydrin was the method of choice Corfield and Robson (1962) developed an automatic technique for the polarographic estimation of amino acids as their copper complexes. Citrulline was used as an internal standard. However, the sensitivity of this technique was found to be no more than the ninhydrin method.

A single column of high resolving power was utilized by Hamilton (1963) in a fully automatic procedure to separate amino acids in 21 hours. Quantities as low as 10^{-8} mole could be determined with a packing resin of 17.5×10^{-4} cm in diameter. Krampitz and Wieneke (1963), however, described a multianalyzer which was capable of doing 6 complete analyses simultaneously. The separation was effected on an Amberlite column and the quantities were estimated by the ninhydrin method.

The next major advance was a further increase in speed and resolution by the use of small but spherical resin particles. Thus Benson and Patterson (1965 a, b) were able to analyze 3 hydrolyzates per day by using an accelerated automatic chromatographic technique on a spherical resin. The average accuracy and precision in the 0.1 to 0.5 micro-mole sample range was 100 $\pm$ 3 percent.

Active charcoal columns also were used by Sleim and Messiha (1966) to separate the aromatic amino acids and the basic amino acid picrates in protein hydrolyzates. Elution of the former was effected by aqueous ethylacetate; histidine, arginine, and lysine with pyridine,

hydrochloric acid and ammonia, respectively.

Continuous unattended analyses of 6 samples were possible in 24 hours, using two rotary valves in automatic amino acid analysis. One valve was used to regulate the 4-buffer system, the other to control the column selection (Dus et al., 1966).

Mondino (1967, pp. 100-112) described a new system of automatic amino acid analysis based on ion exchange chromatography. He used new syringe-type pumps and recording colorimeters which were linear with respect to optical density, so it was possible to evaluate the chromatograms simply by measuring their peak height. A total analysis time of less than 170 minutes was required. The sensitivity of the technique was such that quantities as low as a few tenths of a nanomole could be determined easily. Using this system it was possible to perform three analyses in an 8-hour day and the analyzer was necessarily left idle overnight.

The ultimate degree of sophistication yet reached seems to be in the instrumentation of Eveleigh et al. (1967). These investigators described a completely automatic amino acid analyzer which was capable of analyzing 12 protein hydrolyzates or 4 serum samples per day. Their system included a set-up for sampling which could be loaded with up to 40 samples, these samples were automatically transferred, in sequence, to the ion exchange columns. Thus not only was there automatic chromatography but also automatic loading of samples so that the instrument could be in continuous day and night operation.

In the present work a "second generation" Amino Acid Analyzer

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Phoenix, Model K-8000 based on the design of Spackman _et_ _al_. (1958) was used. Normally advances in knowledge are able to be used immediately, however advances in methodology can only be used when equipment is replaced; thus this analyzer while functional for many years to come is obsolescent by current standards.

Hydrolytic Procedures

To determine the amino acid content of food proteins, or any proteins for that matter, one must first hydrolyze them into their amino acid components. These are then separated and analyzed quantitatively. With the advance of science and technology highly specialized and elaborate instruments for the analysis of amino acids are now available. These instruments have a high degree of accuracy, thus it is no more the actual assay which is the weakest link but the conditions governing the hydrolysis. The question of the best hydrolysis time, temperature, reagent, etc., for the assay of each and every one of the amino acids has not yet been completely and satisfactorily resolved. There are several procedures available depending on the accuracy required and the purpose for the hydrolysis (Block and Weiss, 1956, pp. 18-31; Hill, 1965, pp. 37-99). These include: (a) enzymatic hydrolysis (b) alkaline hydrolysis (c) acid hydrolysis. Of these acid hydrolysis is by far the most commonly adopted procedure.

It is known that hydrolysis in large volume of acid would minimize losses of amino acids. Thus Macpherson (1946) hydrolyzed proteins under reflux by first heating them in a boiling water bath

with 10 volumes of 11 N HCl until they dissolved and then lowering the concentration of HCl to 6N upon addition of distilled water. The time period required was 24 hours. Stein and Moore (1948) found that under dilute conditions the amount of humin formed during hydrolysis was reduced markedly.

Schram et al. (1953, p. 53) hydrolyzed food proteins - an amount equivalent to 8 mg nitrogen - by heating at 135 to 140°C, using an oil-bath, in 200 ml 6N HCl under reflux for 24 hours.

Food proteins are usually associated with carbohydrates. To study the effect of carbohydrates on amino acids during hydrolysis Dustin et al. (1953) prepared a synthetic mixture of 15 amino acids excluding cystine, methionine, and tryptophan, which was boiled for 22 hours in 6N HCl under reflux with and without addition of carbohydrate. Upon analysis by ion exchange chromatography it was found that "in no instance did the addition of starch or glucose (2 gm) to 25 to 50 mg amino acids per 200 ml 6N HCl lower the observed recovery of an amino acid by as much as 3 percent". Later there was good evidence for decrease in loss of amino acids with large excess of HCl (Tristram and Smith, 1963, p. 232).

Smith and Stockwell (1954, pp. 501-513) hydrolyzed pure proteins in 500 volumes of 6N HCl at 105°C. They found that by evacuating hydrolysis tubes to 12 mm Hg little or no humin was formed. The excess HCl was removed in vacuo at 40 to 50°C.

Wahlroos and Land (1959) hydrolyzed milk protein after it was dialyzed, freeze dried and extracted with ether. However, this was possible because the carbohydrates of milk are soluble; this is not

so in the case of foods containing starch.

A more extensive study on hydrolytic procedure was reported by Kimmel et al. (1959). They hydrolyzed pure proteins with 500 times by weight of three times glass-distilled 6N HCl in sealed tubes under vacuum at 110°C for 20 or 70 hours. The protein content of hydrolyzates prepared was usually about 2 mg per ml. Aspartic acid and threonine exhibited destruction in acid hydrolysis. The losses, however, were corrected for by plotting the yield of each amino acid against the time of hydrolysis and extrapolating to zero time. The degree of destruction after 20 hours was 5 percent for each. There was no significant destruction of serine. The yields of valine, leucine, histidine and arginine were significantly higher when hydrolysis was done for 70 hours. It was reported also that the amount of ammonia present in acid hydrolyzates of proteins increased with time of hydrolysis. This, according to the authors, was associated with the destruction of certain amino acids particularly threonine and serine.

Further studies on hydrolysis of proteins were reported by Krampitz (1960) who pointed out that:

> "With a large substrate to acid ratio, the breakdown of free amino acids, excepting tryptophane, methionine and cystine/cysteine, was not affected by the presence of large quantities of carbohydrates in 24 hour hydrochloric acid hydrolysis. Protein-bound amino acids (bovineserum albumine) in the presence of large quantities of different carbohydrates exhibited no breakdown under HCl-hydrolysis. Exceptions were tryptophane, methionine and cystine/cysteine."

The author further noticed that the production of brown color by the hydrolysis of carbohydrate-rich protein carriers could be minimized

by using dilute HCl with the addition of $SnCl_2$.

Another approach taken to investigate the conditions of hydrolysis was to analyze known mixtures of amino acids before and after hydrolysis. Destruction was found to be unavoidable specially in the case of proteins associated with carbohydrates, which would accelerate decomposition of some amino acids (Tristram, 1946; Rees, 1946; Tristram, 1949). One limitation to this approach was that in hydrolysis free amino acids behave differently from peptide - or protein-bound amino acids, as exemplified by Krampitz's studies.

Later on Tristram and Smith (1963, pp. 231-239) emphasized the necessity to treat each protein differently, putting a heavy burden upon the hydrolytic procedure used for amino acid analysis. For accurate analyses, they claimed, due attention had to be paid to the conditions of hydrolysis; different time periods were necessary; also for those amino acids known to be subject to destruction extrapolation to zero time was to be employed. The committee on the Evaluation of Protein Quality (Pellett, 1963, p. 5) while recognizing that more work was needed on hydrolytic procedures for foodstuffs quoted that destruction of amino acids during hydrolysis could be minimized by using a large volume of HCl (12 mg or less nitrogen in 300 ml 6N HCl) and under N_2. This procedure was followed in this study.

More work on hydrolysis in the presence of carbohydrates was reported by Gordon and Basch (1964). A mixture of β-lactoglobulin and starch having a similar composition to a carbohydrate-rich food (2 to 4 mg β-lactoglobulin and 40 to 80 mg starch) was hydrolyzed in 4 to 8 ml

6N HCl in evacuated sealed tubes for 24, 72, and 96 hours at $110^{0} \pm 1^{0}C$. It was found that the presence of carbohydrate had no destructive effect upon aspartic acid, threonine, serine, glutamic acid, proline, alanine, valine, isoleucine, leucine, phenylalanine, lysine, and histidine. Glycine exhibited a small apparent destruction which was thought to be insignificant. Cystine and methionine were destroyed to a greater degree, which would necessitate the use of a different treatment for their estimation. Tryptophan also showed a destruction of about 25 percent under the conditions of the experiment and thus required a different procedure for its estimation. The presence of starch caused a serious destruction of tyrosine (75 percent recovery). Lyman <u>et al</u>. (1958) had claimed that this could be minimized by adding excess tryptophan before acid hydrolysis; this was confirmed by Gordon and Basch (1964), but because of the extra humin produced acid hydrolysis under reflux with dilute solutions was suggested as a preferable procedure.

The possibility of interference of fats, carbohydrates, and salts in amino acid determinations were investigated by Smith <u>et al</u>. (1965) in fish meals, fish protein concentrates, and mixed animal diets. In general, none was found to have any destructive effect on amino acids, with acid hydrolysis (6N HCl) under reflux for 24 hours. With increase in time of hydrolysis (24, 48, 72 hours) the amino acid content of fish meal and fish protein concentrate decreased.

As pointed out earlier, hydrolysis under N_2 resulted in further reduction in destruction of amino acids. Thus hydrolysis of feeds with 5.7 M HCl in sealed tubes in an atmosphere of N_2 or in an HCl

vapor atmosphere as well as hydrolysis under reflux in 5.7 M HCl with no attempt to remove air were reported by Finlayson (1965). The samples (about 500 mg each) in sealed tubes were hydrolyzed in an oven at 107°C for 6, 10, 20, 40, and 70 hours. The hydrolyzates were filtered to remove any precipitate, evaporated and taken up in 0.1 M HCl for analysis. Results showed a progressive decrease in recoveries of serine, threonine, cystine, methionine, valine, and isoleucine as the hydrolysis time exceeded 10 hours. Hydrolysis periods of 40 to 70 hours caused relatively big losses of serine (21 to 32 percent), threonine (16 to 24 percent) and cystine (more than 50 percent). Methionine showed more stability in prolonged hydrolysis (10 to 15 percent loss after 70 hours). The other amino acids were stable under the conditions of the experiment. The 10-hour hydrolysis, though it lead to maximum recovery of most amino acids (serine, threonine, cystine), was not sufficient to give maximum valine and isoleucine recoveries. Hydrolysis under reflux without replacing air caused an additional loss of serine (5 percent) and threonine (10 percent) in 20 hours as compared to sealed tube hydrolysis with the same period. The ratio of sample size to the amount of acid was found to be important. A 3 to 4 percent higher recovery was reported for 2 mg feed per ml as against 20 mg per ml of acid.

Jacobs (1966, pp. 190-196) believed that optimum hydrolysis conditions varied from protein to protein, or from peptide to peptide, and that each substance had to be investigated for the best hydrolytic conditions. Despite this the author found it more convenient to

hydrolyze most proteins in sealed Pyrex glass containers with 6N HCl, under N_2, at 105°C for 24 hours. The N_2 gas was bubbled into the containers before sealing to replace acid, hence the oxidation of methionine to methionine sulfoxides and methionine sulfone was minimized.

In the present work the hydrolytic procedure involving large volume of 6N HCl in a nitrogen atmosphere, under reflux, which was adopted in most of the investigations already reviewed, was the method of choice.

Sulfur Amino Acids

The limiting amino acids in most food proteins are the sulfur amino acids (cystine/cysteine and methionine) which unfortunately are amongst those most liable to destruction during hydrolysis. Hence it is particularly important to determine the amount of these amino acids under conditions of minimal destruction. An ideal way, perhaps, would be to measure them while they are still attached in a peptide form, but no such method has yet been developed. Many methods have been reported for estimating the cystine content of proteins (Block and Bolling, 1951, p. 183). A preoxidation procedure by performic acid followed by hydrolysis has been used by many workers.

Toennies (1942, pp. 667-677) oxidized casein to convert methionine into methionine sulfone and noticed that cystine disappeared in part. Sanger (1949) applied the performic acid oxidation reaction "to split -S-S- bridges of insulin as the first step in investigation of structure of the hormone".

In 1954, Schram et al. emphasized that estimation of cystine content of proteins is mostly complicated by the instability of cystine and cysteine during hydrolysis particularly in the presence of carbohydrates. Hence they determined cystine as cysteic acid; the protein was first oxidized with performic acid, this was followed by 20 hours of HCl (6N) hydrolysis. Under the experimental conditions used, cystine and cysteine gave 90 ± 2 percent of the theoretical yield of cysteic acid. When they applied the method to proteins, the cystine content (i.e. actually measured as cysteic acid) was increased by an average of 10 percent. Furthermore, since performic acid must be made freshly by mixing hydrogen peroxide with formic acid for each oxidation, the conditions of its admixture could affect the results obtained. These authors showed that increasing the concentration of hydrogen peroxide in the formic acid did not have any effect on the final cystine recovery, neither did increasing the reaction time, oxidation at -10^{0}C, nor removal of the excess performic acid by freeze drying.

The enzyme ribonuclease was oxidized with performic acid (Hirs, 1956, pp. 611-620) under different conditions and the amino acid composition of the native protein was compared with that of the oxidized protein. Chloride-free ribonuclease oxidized at 0^{0}C incurred transformation of cystine to cysteic acid and methionine to methionine sulfone. No other amino acids were significantly affected. To avoid the formation of chlorotyrosine, oxidation at -10^{0}C was necessary when traces of chloride were present.

Bidmead and Ley (1958), however, followed the example of Schram

et al. (1954) and used two separate analyses upon hydrolyzates with and without preoxidation for determining the amino acid composition of food proteins. Cystine and methionine had a 100 ± 3 percent recovery, the rest suffered a loss of approximately 10 percent. Phenylalanine, tyrosine, and histidine had losses as high as (or higher than) 50 percent. The use of the freeze dryer rather than the rotary evaporator for the removal of the performic acid resulted in much lower losses; this indicated that the losses were mostly during the removal of the performic acid.

According to Tristram and Smith (1963, p. 240) it was necessary that the total number of half cystine and cysteine residues be determined by independent analyses on additional protein samples, implying destruction under ordinary hydrolytic procedures.

A modified method of Schram *et al*. (1954) was developed by Moore (1963) for the determination of cysteine and half cystine as cysteic acid. In this procedure a reducing agent (HB_r) was added at the end of the oxidation to destroy the excess performic acid, thus preventing overoxidation and consequent losses during the evaporation to dryness stage. It was claimed that this also provided a method for the measurement of methionine as methionine sulfone. Further application of the method to protein mixtures containing up to 94 percent carbohydrate met with success. The chromatographic technique (Moore, 1963) for the separation of these oxidized amino acids was also modified by Bujard and Mauron (in Pellett, 1963, p. 5) in such a way that it was possible to determine both cystine and methionine as cysteic acid and methionine sulfone, respectively, ahead of all

the other amino acids.

Cystine content of some 22 samples of different foodstuffs were reported by De Belsunce and Pion (1963). Performic acid treatment at -10°C for 4 hours resulted in recoveries of cystine as cysteic acid with values which were claimed to be in agreement with the published results obtained through other methods.

Later Finlayson's studies (1965) involving the oxidation procedure of Hirs (1956) revealed an increase of 10 to 15 percent in cystine, recovered as cysteic acid.

In the present investigation the performic acid oxidation procedure of Moore (1963) as modified by Bujard (Personal communication, see Appendix A) was used to estimate both cystine and methionine.

III. MATERIALS AND METHODS

Amino Acid Analysis

<u>Selection of samples:</u> The main criterion for selecting samples was whether the particular item could in fact supply sufficient protein to the consumer's daily intake to make the consideration of its amino acid composition meaningful. Thus both low protein sources which are consumed in considerable quantities and high protein sources which may only be taken infrequently and in small amounts have been included.

<u>Sample preparation:</u> Ordinary drying procedures using high temperatures are conducive to protein damage, thus freeze drying was used to dry the food samples.

<u>Freeze drying:</u> A small laboratory vacuum dryer was modified in such a way to make it possible to cope with relatively large quantities of food materials (Figure 1). This freeze dryer was composed of a vacuum pump, a vacuum chamber with a thermostatically controlled heater, and a freezer section. Two big batches of activated aluminum oxide granules (supplied by Peter Spence and Sons Ltd. Widnes) were put in two perforated steel baskets which were manufactured in the workshop (School of Engineering, A.U.B.). One of the baskets was placed in the vacuum chamber and the other in the freezer unit. The purpose of putting these desiccants was to increase the drying rate by absorbing much of the moisture released during the

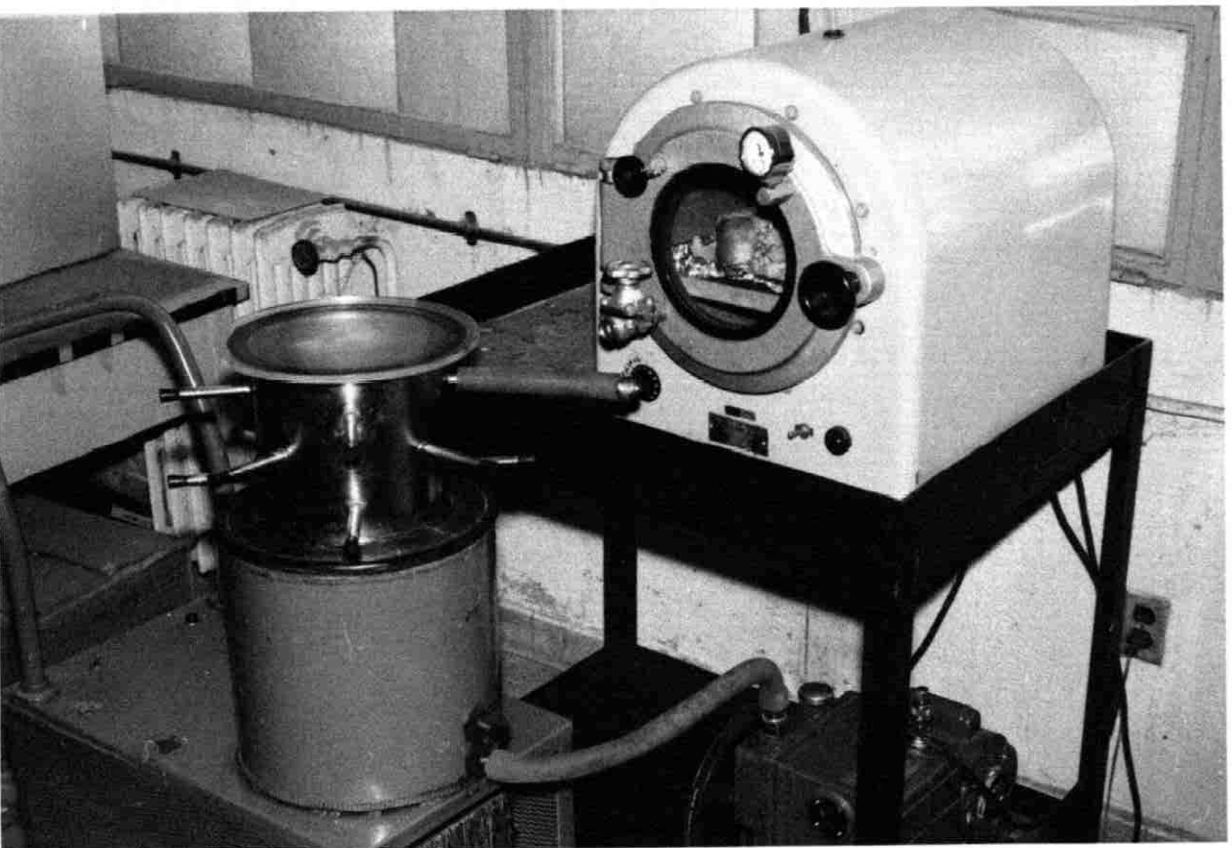

Figure 1. Laboratory freeze dryer.

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course of drying. The aluminum oxide granules could be regenerated by heating to 260°C for 3 to 4 hours (Payne et al., 1961).

Reasonable amounts of each sample (100 to 200 gm wet weight) were freeze dried by means of the above freeze dryer. Obviously the items that were already dry did not require this process. The freeze dried samples were ground to a fine powder by an electric mixer. They were then kept in small, tightly closed sample jars until analyzed.

Preparation of standard amino acid mixture: A standard amino acid mixture was prepared in 0.1 N HCl such that it contained 2.5 uM/ml of each of the following amino acids: DL-aspartic acid; DL-threonine; DL-serine; L-glutamic acid; L-proline; glycine; DL-alanine; L-cystine; DL-valine; DL-methionine; L-isoleucine; L-leucine; L-tyrosine; DL-phenylalanine; L-lysine; L-histidine; L-arginine; L-cysteic acid; DL-methionine sulfone; DL-methionine sulfoxides. This standard solution, the total volume of which was 250 ml, was stored in 10-ml ampoules in a deep freeze. For analysis a 1 to 5 dilution was prepared by adding a solution of citrate buffer pH 2.2 (Appendix D) ; 2-ml aliquots containing 1 uM of each amino acid were analyzed with each new batch of the ninhydrin reagent. Standard solutions of ammonia (prepared from $(NH_4)_2 SO_4$), nor-leucine, and taurine were also prepared separately and stored in a similar manner. The reliability of these standards was confirmed by comparing against a standard mixture which was purchased from Phoenix Chemical Company (Appendix B).

Hydrolysis: To prevent errors due to changes in moisture content

during storage, duplicate samples were weighed, one for the determination of N content and the other to be hydrolyzed for amino acid analysis. A quantity of the sample containing about 12 mg nitrogen was accurately weighed on a UNIMATIC analytical balance. It was then transferred into a 1000-ml round-bottom flask; any traces left on the weighing paper were washed into the flask with distilled water from a wash-bottle. Then 300 ml of 6N HCl were added and the whole preparation was boiled for 24 hours under reflux on a GLAS-COL electric muntle heater (Figure 2). A very slow stream of purified N_2 (Appendix C) was passed through the boiling acid. At the end of hydrolysis period the hydrolyzate was cooled to room temperature, filtered through a medium porosity sintered-glass funnel of suitable size using a suction pump. The purpose of this filtration was to remove humin, the decomposition product of tryptophan, and the other brown-colored materials resulting from the decomposition of carbohydrates and other components of the material being analyzed. A slightly brown-colored filtrate was collected, evaporated to dryness on the Craig rotary evaporator (Figure 3), washed twice with a few ml of distilled water and re-evaporated to dryness. This was then dissolved in a few ml of sodium citrate buffer pH 2.2 (Appendix D), and filtered through Whatman NO.4 filter paper to remove any further residue. The volume was ultimately made up to 25 ml and kept under N_2 gas in a deep freeze until analyzed.

Chromatography: Aliquots of the protein hydrolyzates (usually 0.5 ml) were analyzed on an automatic Amino Acid Analyzer, Phoenix, Model K-8000 (Figure 4 and Appendix E) based on the method devised by

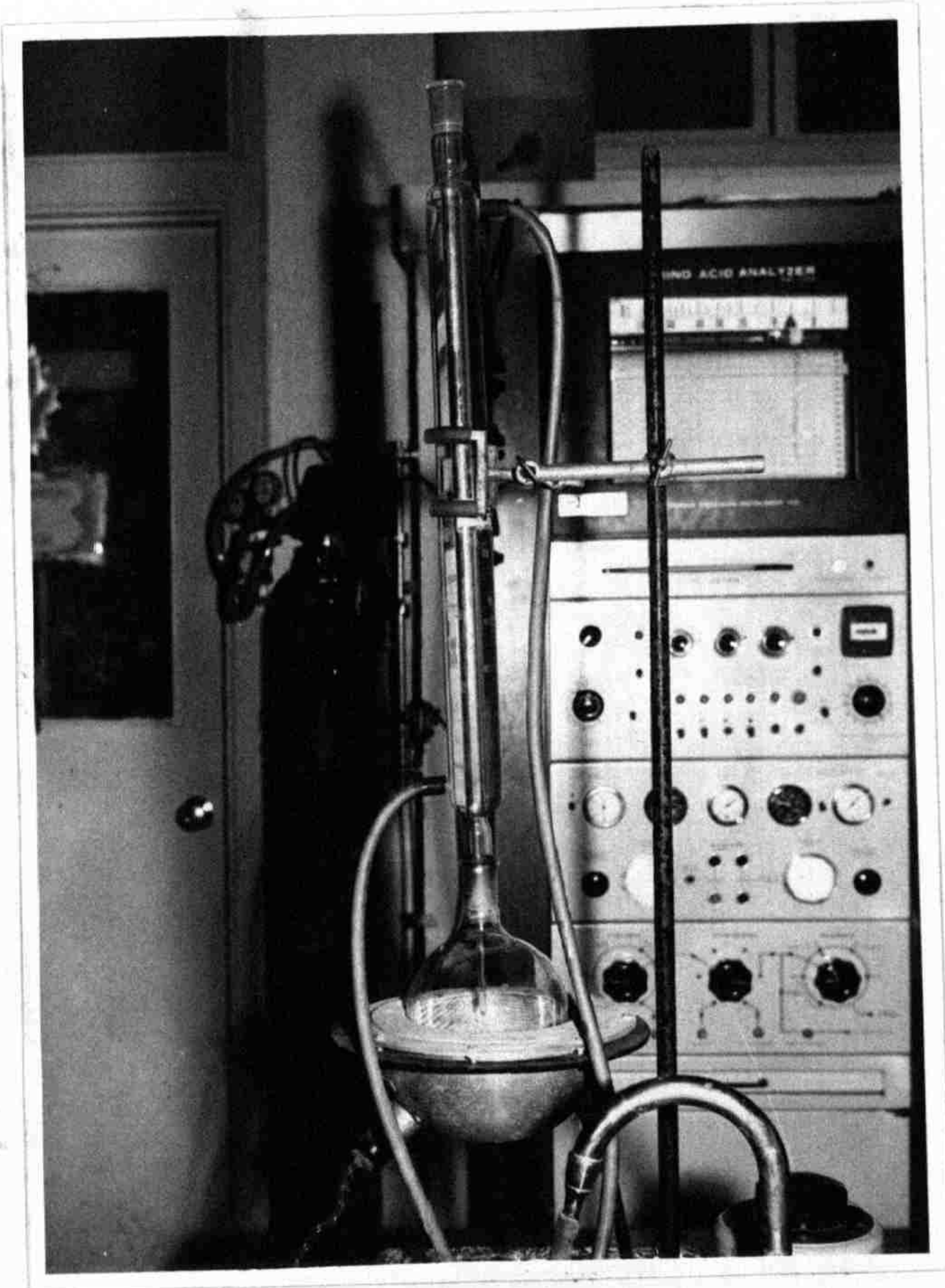


Figure 2. Hydrolysis apparatus.

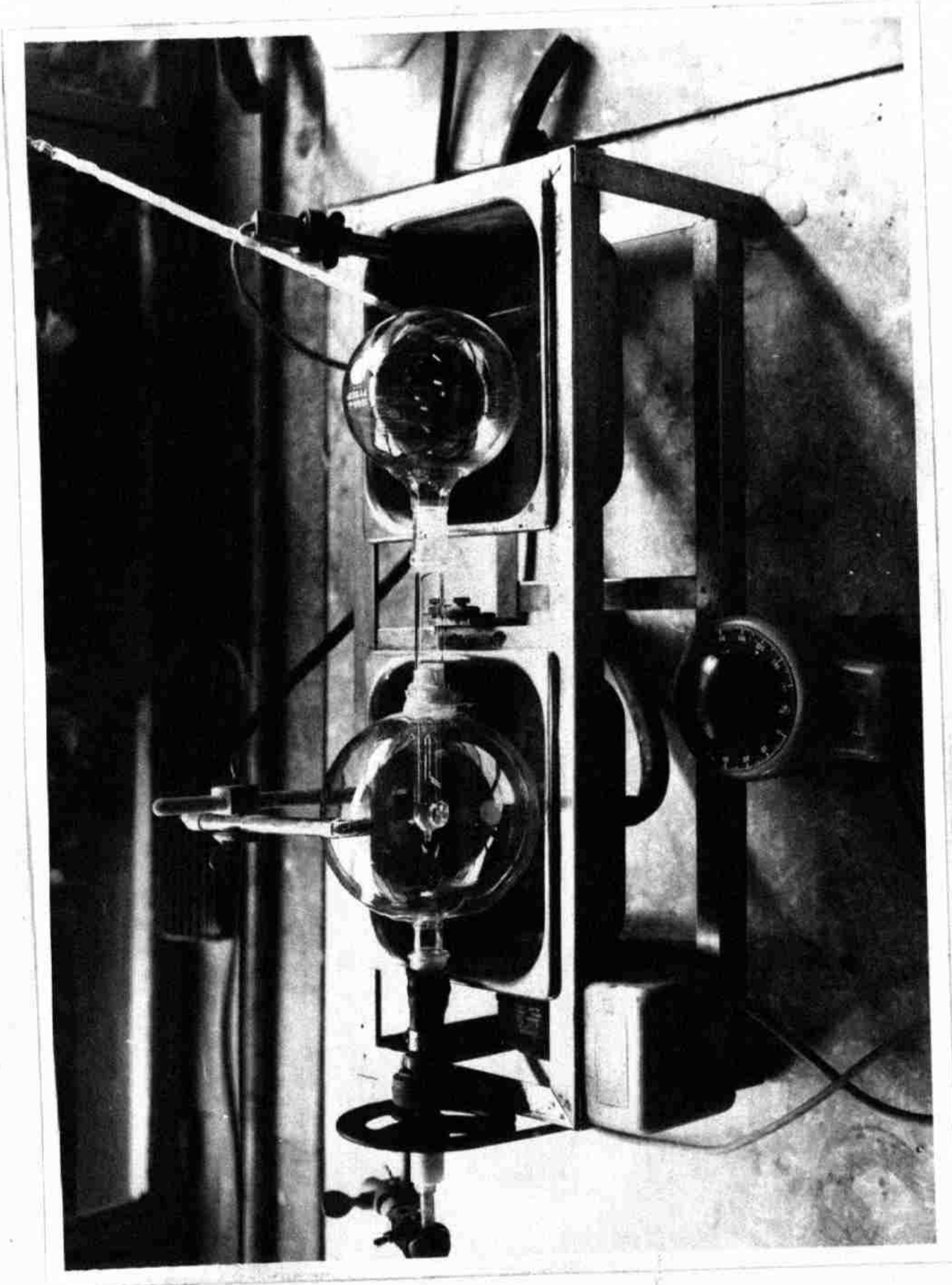


Figure 3. Craig rotary evaporator.

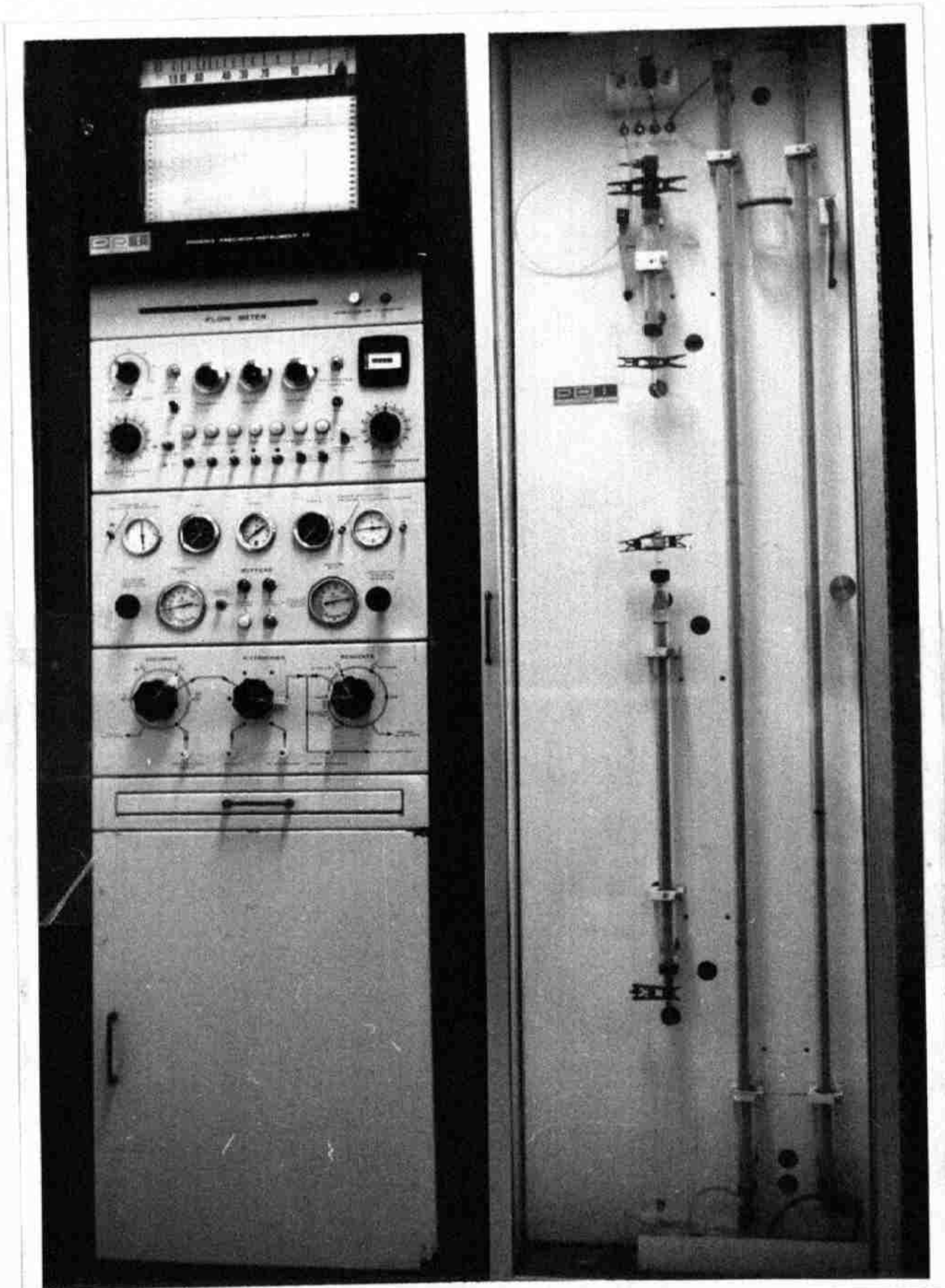


Figure 4. Automatic Amino Acid Analyzer.

Spackman et al. (1958, pp. 1190-1206). Two types of water-jacketed columns packed with resin were utilized for the separation of the amino acids. One short column (15 X 0.9 cm) was used to separate the basic amino acids (lysine, histidine, arginine, and "ammonia"); the two long columns (150 X 0.9 cm) were used alternately for the acidic and neutral amino acids (cysteic acid, methionine sulfoxides, aspartic acid, methionine sulfone, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, nor-leucine, tyrosine, and phenylalanine).

Integration and interpretation of chromatograms: The amino acids present in the hydrolyzates were chromatographed as a series of peaks (Figure 5), the areas of these peaks were proportional to the quantities of the amino acids analyzed. A simplified method was used for calculating the peak areas (Phoenix Instruction Manual). This involved counting the number of dots, recorded at fixed time intervals around the upper half of the curve and multiplying by the net height of the peak. The values so obtained were compared with those for similar peaks for a standard solution containing one micromole quantities of each amino acid, chromatographed under the same conditions. The ratio of the peak area calculated for the unknown quantity of an amino acid to that of standard was the amount in micromoles present in the aliquot analyzed. Using a dilution factor and considering the exact amount of nitrogen contained in the total volume of the hydrolyzate as well as the molecular weight of each specific amino acid, the amount in mg/gm nitrogen was calculated (for more details see Appendix F) as follows:

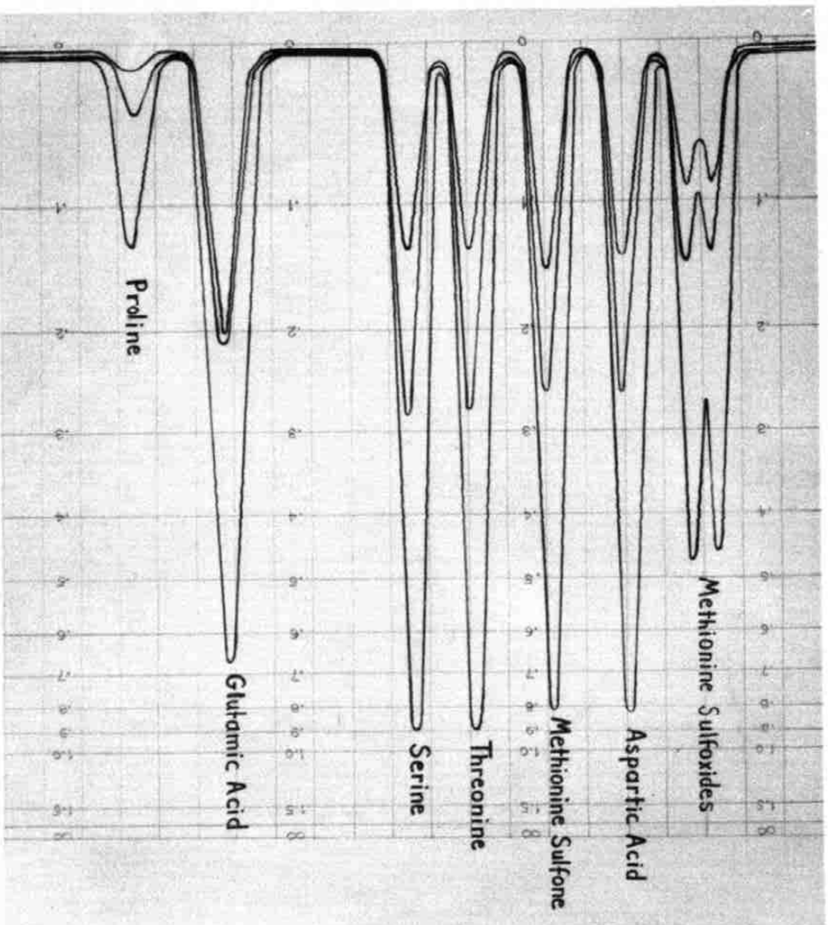


Figure 5. Part of a representative chromatogram.

$$\frac{\frac{H \times W}{C} \times M.W. \times F}{Wt.N} = \text{mg amino acid/gm N}$$

H = Net height of the peak.
W = Number of dots around the upper half of the peak.
M.W. = Molecular weight of amino acid.
F = Dilution factor, considering the total volume of the hydrolyzate and the aliquot analyzed.
Wt.N = Total amount of nitrogen (mg) present in the hydrolyzate.

Sulfur Amino Acids

Sulfur amino acids, cystine/cysteine and methionine, are the limiting amino acids in most world dietaries and are thus frequently the limiting amino acids in many foodstuffs; this is even more so in the protein nutrition of the economically developing countries. "In these countries protein frequently must be provided in the most economical form possible, and the amount available is often so limited that quality or biological value assumes an unusual importance" (Barnes, 1963, p. 2). This necessitates the knowledge of the exact amount of sulfur amino acids in food proteins. Unfortunately these amino acids, as mentioned earlier, are known to be partially destroyed upon ordinary hydrolysis and thus have to be determined independently of the other amino acids.

Several methods have been proposed, of these the one most widely used is that involving the preoxidation of samples under investigation with performic acid and their subsequent acid hydrolysis. This oxidation process will convert cystine and methionine to cysteic

acid and methionine sulfone, respectively, which are the more stable forms and can be recovered quantitatively. In the course of the present study a modified method of Moore (1963) as described by Bujard (personal communication, Appendix A) was used.

Preoxidation with performic acid and hydrolysis:

Preparation of the reagent: The performic acid reagent was prepared as follows: To 72 ml of 98 to 100 percent formic acid were added 1.5 ml methanol (to prevent freezing) and 7.5 ml of 30 percent H_2O_2. The mixture was kept at room temperature for 2 hours in a closed vessel for maximum performic acid formation.

Procedure: A sample equivalent to 7 mg Kjeldahl nitrogen was weighed and transferred to a 500-ml ground-neck, flat-bottomed flask containing a few drops of the freshly prepared performic acid. The mixture was cooled to -10^0 to -14^0C for 30 minutes. Twenty five ml of the performic acid were then added and the sample was refrigerated (at 4^0C) overnight. The volume was doubled with distilled water and 2 ml of 48 percent HBr (to remove any extra performic acid and avoid overoxidation) were cautiously added. Finally, it was concentrated at a temperature of 40^0C on the rotary evaporator containing about 20 ml of 1N NaOH in the condenser flask to absorb the HBr as it distilled over. Next the oxidized, concentrated material was hydrolyzed with 100 ml of 6N HCl for 24 hours, as described under "Hydrolysis", the only difference being in the volume of HCl used and the absence of N_2 bubbles since the sample was already fully oxidized. The rest of the process of hydrolysis, i.e., filtering, concentrating, and dissolving in buffer solution was done exactly as before.

Chromatography: Aliquots of the preoxidized hydrolyzates were subjected to analysis with the Amino Acid Analyzer using the 150-cm columns. Analysis was continued, on a number of early runs, as far as the emergence of methionine sulfone peak (about 5½ hours). Later, for reasons which will be discussed in a subsequent section, the analysis time was extended to 12 hours which was long enough for the emergence of methionine. In practically all these later analyses some intact methionine was recovered.

Determination of Tryptophan

Tryptophan is completely destroyed during acid hydrolysis, thus alternative hydrolytic procedures are needed for its measurement. These include: alkaline hydrolysis (Horn and Jones, 1945; Drèze, 1956; Kofrányi, 1964; Tkachuk, 1966), and enzymatic hydrolysis (Spies and Chamber, 1948, 1949, 1950; Scott, 1961; Lombard and De Lange, 1965) with subsequent analysis either by column chromatography (Tkachuk, 1966), or electrophoretically (Wapnir and Bessman, 1965) or microbiologically (Sebeck, 1965).

A modified procedure of Lombard and De Lange (1965) was applied for the determination of tryptophan in the samples analyzed. Modification was needed because the method as proposed by these authors, requiring an internal standard, did not give consistent results, thus a procedure involving the construction of a standard curve was adopted.

Reagents and apparatus: Enzyme solution freshly prepared by shaking 2 gm of papain (Merck) with 100 ml of H_2O for 2 min., and filtered

before use; 5 percent solution of p-dimethylaminobenzaldehyde in conc. HCl (A.R.); 5 percent aqueous solution of sodium cyanide; 0.5 N aqueous solution of sodium hydroxide (A.R.); 0.1 N aqueous solution of KOH (A.R.); freshly prepared 0.2 percent aqueous solution of $NaNO_2$; conc. HCl (A.R.); carbon tetrachloride (A.R.); tryptophan standard solution (10 mg tryptophan dissolved in distilled H_2O and as much conc. HCl as required (around 6 drops) and diluted to 100 ml; 1 ml of standard solution contains 100 micro-gm tryptophan); Zeiss, Unicam, Beckman, or Bausch and Lomb spectrophotometer; Adams centrifuge; Griffin flask shaker; 90-ml flat glass bottles with metal caps having rubber seals; 125-ml ground-neck Erlenmeyer flasks; water bath; 15-ml Pyrex centrifuge tubes; Vortex Jr. test tube mixer; suction bulb.

Procedure: Two different weights of each sample containing approximately 600 to 700 mg protein were measured into two 90-ml flat-bottomed bottles. Twenty five ml of 0.05 N NaOH, 10 ml of enzyme solution and 10 drops of 5 percent aqueous sodium cyanide solution were then added to each bottle. For the determination of the tryptophan content of the enzyme, 25 ml of the NaOH solution were mixed with 30 ml of the enzyme solution and 10 drops of the NaCN solution were added. For every set of samples one such determination was needed. The containers were sealed, with screw type metal caps having rubber seals. After an overnight digestion in a water bath at $70 \pm 1^{\circ}C$ they were cooled, made up to the 90-ml mark with distilled water, mixed thoroughly by shaking and allowed to settle. To 5 ml of each hydrolyzate obtained at this stage were added 5 ml of 0.1 N KOH

and 3 ml of carbon tetrachloride in a 125-ml Erlenmeyer flask. The flask was stoppered with an E-mil plastic stopper and shaken for 10 minutes on a Griffin flask shaker. The top layer was transferred to 15-ml centrifuge tubes and centrifuged for 10 to 20 minutes at 3000 r.p.m. One ml of the clear supernatant fluid was pipetted into each of 3 test tubes, two marked test (T) and one blank (B). One ml of p-dimethylaminobenzaldehyde (5 percent in conc. HCl) was pipetted into test tubes (T) using a suction bulb; one ml of distilled water was added to (B). Then 5 ml of conc. HCl were added to all three test tubes. They were shaken on a Vortex Jr. test tube mixer. After 10 minutes 2 drops of freshly prepared 0.2 percent $NaNO_2$ were added to each, shaken for 5 seconds and let stand for 5 minutes for color development. The intensity of the color formed (stable for 1 hour) was read at 590 mu against the blank (B) in a spectrophotometer (Spectronic 20, blue filter). The optical densities were interpreted by comparing with the similarly obtained values for graded quantities of standard tryptophan solution (0 to 75 micro-grams, with 5 unit intervals). One ml of each concentration level was transferred to each of three test tubes undergoing all the steps mentioned before. The following formula was applied in calculations:

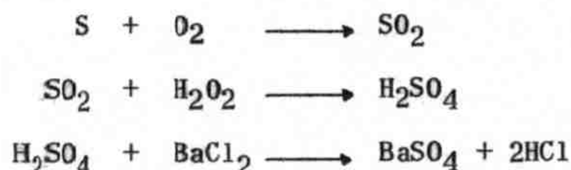
$$\frac{TrS - TrE}{Wt.S} \times \frac{100}{\%N} = \text{mg tryptophan/gm N}$$

TrS = Total tryptophan in the sample = Concentration corresponding to O.D. of unknown (read from standard curve) multiplied by a dilution factor.

TrE = Total tryptophan in the enzyme = Concentration corresponding to O.D. of enzyme (read from standard curve) multiplied by a dilution factor.

Determination of Sulfur

Principle: Food samples were oxidized by combustion (Pellett and Eddy, 1964) and thus the sulfur was also oxidized to sulfur dioxide which was absorbed in hydrogen peroxide, producing sulfuric acid. To this solution was added a solution containing barium chloride, gelatin and dilute HCl; this gave a colloidal precipitate of barium sulfate which was estimated turbidimetrically (Pellett, 1965).



$$S + O_2 \longrightarrow SO_2$$

$$SO_2 + H_2O_2 \longrightarrow H_2SO_4$$

$$H_2SO_4 + BaCl_2 \longrightarrow BaSO_4 + 2HCl$$

The comparison of this simpler and shorter procedure with the barium chloranilate method of Pellett and Eddy (1964) will be discussed elsewhere (Pellett et al., 1968). The method described here had a correlation coefficient of 0.98 with the barium chloranilate procedure.

Apparatus: Boiling tubes; 250-ml quickfit conical flasks; quickfit stoppers fitted with platinum spiral set in glass rod; pipetting syringe (10-ml capacity); volumetric flask (100-ml); colorimeter fitted with red filter or set at a wavelength of 665 mu; dialysis tubing; electric stirring machine.

Chemicals: K_2SO_4; H_2O_2; Gelatin; $BaCl_2$; Conc. HCl.

Preparation of reagents:

$BaCl_2$ reagent: Three gm of gelatin were dissolved in 400 ml of boiling water. After cooling it was poured into a suitable length of dialysis tubing. The ends were tied and the tube was suspended overnight in a large container filled with distilled water and placed on a magnetic stirrer. (The sulfate ions present in gelatin which could give high blank values were thereby dialyzed out). The tube was then emptied and the solution was further diluted to about 700 ml. Ten gm of $BaCl_2$ and 8.5 ml of conc. HCl were added. The preparation was stirred to dissolve the salt and the volume was made up to 1 liter with distilled water.

Standard solutions: 1.09 gm of dried potassium sulfate were accurately weighed and dissolved in distilled water and made up to 100 ml. Aliquots were taken and suitable dilutions were made to give 10, 20, 30, 40, and 60 ug S/ml.

Procedure: A quantity between 130 and 140 mg of dried sample was accurately weighed into half a cigarette paper and folded carefully. Ten ml of distilled water and 2 drops of hydrogen peroxide were added to a quickfit conical flask which was then filled with oxygen flowing for about 15 seconds from an oxygen cylinder. The flask was immediately stoppered with a rubber stopper. The sample, inserted firmly into a platinum spiral, was briefly held in a flame till the paper started burning. The rubber stopper was removed and the burning sample was quickly plunged into the flask of oxygen, the flask being closed with the attached stopper. The sample burnt with a bright orange flame. If any pieces of black material were present in the

flask after the flame died down, or in case there was a thick sooty deposit, the sample was discarded and the combustion repeated with a smaller weight of food sample. The burnt sample was allowed to stand for half an hour with occasional shaking. Ten ml of the reagent were added, and filtered through folded cheese cloth into test tubes. Optical densities were read immediately at 665 mu on a spectrophotometer (Spectronic 20, Bauch and Lomb). A standard curve was obtained by adding 10 ml of the reagent to 10 ml of graded levels of sulfate solution; these were also read on the colorimeter against 10 ml of distilled water plus 10 ml of the reagent serving as blank. The concentrations of sulfur in the food samples were obtained from the standard curve.

Calculations: Let the weight of food be W and the graph reading x (ug/ml). Since 10 ml of sample solution contained x ug S/ml, thus W gm of food contained 10x ug S. Therefore, $\frac{10x \times 100}{W \times 1000}$ = mg S/100 gm sample thus x/W = mg S/100 gm sample.

Determination of Nitrogen

The nitrogen contents of samples were determined by the macro-Kjeldahl procedure (Triebold and Aurand, 1963, pp. 25-31). The protein content, whenever needed, was always calculated by multiplying percent N by 6.25.

Determination of Metabolizable Energy

Gross energy (G.E.) was determined using a ballistic bomb calorimeter according to the procedure of Miller and Payne (1959),

sucrose being used as the reference standard. The metabolizable energy (M.E.) was calculated from gross energy using the equation of the above authors;- $M.E. = 0.95 \times G.E. - 0.075 \times \%N$.

Protein Quality "Scores"

The biological value of a food protein is related to its content of essential amino acids. The relative lack or abundance of these amino acids will determine whether a food protein is good or poor for growth and tissue repairs. So far a number of methods have been proposed which will score food proteins on the basis of their content of essential amino acids (Campbell, 1963). The two procedures recommended by FAO 1957 and FAO/WHO 1965, as well as a sulfur scoring system were used in this study.

FAO 1957 scoring procedure: The provisional amino acid pattern of the reference protein published by the FAO 1957 Committee contained the levels of essential amino acids shown in Table 1. Out of the eight essential amino acids only four (lysine, tryptophan, sulfur amino acids, and threonine) are known to be potentially limiting in human foods. Comparison was made between the amount of each of these four essential amino acids in a protein expressed as percentage of their respective value in the reference protein. The lowest of the four percentages obtained was assigned as the score of that particular protein.

Table 1. Essential amino acids in the FAO 1957 provisional pattern[x].

Amino Acids	mg/gm N
Isoleucine	270
Leucine	306
Lysine	270
Phenylalanine	180
Tyrosine	180
Sulfur amino acids:	
Total	270
Methionine	144
Threonine	180
Tryptophan	90
Valine	270

[x] From Table 3, p. 26, Protein Requirements, FAO, 1957.

FAO/WHO 1965 scoring procedure: The provisional pattern of amino acids in the reference protein reported by the FAO 1957 was found to have limitations. Hence in 1965 the FAO/WHO Expert Group on Protein Requirements published a new recommended method for scoring the quality of a food protein. The values reported by this group were based on the pattern of the essential amino acids in the egg protein as shown in Table 2. Two ratios were described by this committee: (1) the A/E ratio which was the amount in mg of each essential amino

acid per gm of total essential amino acids, (2) the E/T ratio which was the amount in gm of total essential amino acids per gm of total nitrogen.

Table 2. The pattern of essential amino acids in egg protein[x].

Amino Acids	mg/gm total N		Percent of total essential amino acids	
Isoleucine	415		12.9	
Leucine	553		17.2	
Lysine	403		12.5	
Aromatic	627		19.5	
Phenylalanine		365		11.4
Tyrosine		262		8.1
Sulfur amino acids	346		10.8	
Cystine		149		4.6
Methionine		197		6.2
Threonine	317		9.9	
Tryptophan	100		3.1	
Valine	454		14.1	
Total	3215		100.0	

[x] From Table 6, p. 36, Part B, Protein Requirements, FAO/WHO, 1965.

This scoring procedure also took the levels of the four potentially limiting amino acids into consideration. The score was calculated by comparing the A/E ratios obtained for each of these amino acids in the protein in question to the corresponding A/E ratios for egg. These values were expressed as percentages and the lowest was taken as the protein quality score.

Both FAO 1957 and FAO/WHO 1965 scoring procedures were calculated by means of a program written for IBM 1620 computer.

Sulfur score: It was demonstrated by Miller and Naismith (1958) that the sulfur content of mixed dietaries was often a more reliable index of net dietary protein value than was the percentage of nitrogen. At high levels of nitrogen intake, however, the method became unreliable due to the metabolic wastage of protein, caused by diversion of protein into energy pathways. Miller and Donoso (1963) proposed a ratio of sulfur to nitrogen, $(\frac{1000 \times \%S}{\%N})$, as a direct quality index score followed by the use of the nomograph or equation of Miller and Payne (1961a, b) to calculate the net dietary protein value. They showed that there was a close correlation between the net dietary protein values calculated in this way and those obtained by direct rat growth procedures. The ratio was used for British hospital meals by Pellett and Eddy (1964) who also showed good correlation between predicted and determined values for 19 bulked hospital meals. Consequently, the S/N ratio (as well as the ratio of protein calories to total calories) was used by these authors in calculating the protein values of some 450 meals from 150 hospitals.

According to Rose et al. (1954), Miller and Donoso (1963) and

Barnes (1963, p. 2) most human dietaries are limited by their content of sulfur amino acids. Thus the S/N ratio could possibly reflect the protein quality; this assumption was not necessarily true for Middle Eastern diets whose pattern of consumption was in general characterized by a large intake of bread the protein of which would be limited by lysine or threonine rather than methionine. However, a preliminary report by Pellett and Jamalian (1966) indicated that the sulfur prediction was valid at least for some Middle Eastern diets, therefore, it was used in this study and the results will be dealt with later.

Net Dietary Protein as a Percentage of Total Calories

The protein value of mixed diets can be assessed with accuracy through determination of an index called NDpCal percent (Net Dietary Protein as a percentage of total calories). This index combines both quality and quantity of protein with respect to the calorific value of the diet (Platt *et al.*, 1961; Miller and Payne, 1961a, b, c); it can be determined by biological experimentation followed by calculations requiring protein and calorie contents obtained from analysis. An alternative procedure replaces the biological experimentation by straight chemical analyses which lead to protein quality scores. The scores can in turn be used in the formula of Miller and Payne (1961a) to calculate NDpCal percent as follows:

$$\text{NDpCal percent} = \text{PCal percent} \times \text{Score} \times \frac{(54.0 - \text{PCal percent})}{\left(54.0 - \frac{400.0}{\text{Score}}\right)}$$

Where PCal percent = $\frac{P \times 4 \times 100}{M.E.}$

P = Percentage of protein in the diet.

M.E. = Metabolizable energy per 100 gm of the diet.

The value for the score could be either from the FAO 1957 score, the FAO/WHO 1965 score, or S/N ratio.

Check Measures for Accuracy and Reliability of Results of Amino Acid Analyses

In order to ensure that the analytical procedures were accurate and precise and that the results were reliable several independent check measures were applied; some were analytical and some were based on straight calculations as follows:

Nor-leucine internal standard: The synthetic amino acid nor-leucine is never present in protein hydrolyzates. Upon analysis it will be chromatographed as a sharp peak at about 11 hours immediately after the emergence of leucine. One micromole quantities of this compound were analyzed on the long column along with each hydrolyzate. The percent recovery of this amino acid was taken as a criterion to check for any failure during analysis arising from deterioration of the ninhydrin reagent, and also to check for changes in the flow rate of the buffers and/or the ninhydrin reagent. In case the run was grossly erroneous it would lead to an unexpectedly wrong recovery for nor-leucine which would favor rejection of the analysis. However, deviations of plus or minus five percentage units, from the ideal 100 percent, were tolerated and thus appropriate factors were applied to correct the recovery of the rest of the amino acids.

Taurine internal standard: For the chromatography of the preoxidized hydrolyzates nor-leucine could not be used as an internal check since the period of analysis was not long enough to allow for its emergence. Hence it was essential to make use of another amino acid which was normally absent in protein hydrolyzates and could be chromatographed early in the course of analysis. Taurine was found to fulfil both of these requirements. Here again the percent recovery was taken as a reference to decide upon the reliability of the analysis and accordingly apply a factor to correct for small variations. Grossly erratic analyses were rejected. The use of this amino acid had one disadvantage whenever it was analyzed with brown-colored hydrolyzates. The drawback was the superimposition of the taurine peak and the peak for the brown color which would lead to an erroneously high peak area for taurine; the merits, however, justified its use.

Total amino acid nitrogen: A third method which was employed to determine the reliability of the results was to sum up the amount of nitrogen contributed by each amino acid and see whether the total was close to the expected level of 1000 mg. This was done by using the following formula:

$$\text{Total N (mg)} = \Sigma\ (W \times Pn)$$

Where W is equal to the weight of each individual amino acid in mg and Pn the percent N in that amino acid.

The calculations involved were tedious and time-consuming. Thus the quantities of amino acids as well as their respective nitrogen percentages were punched on IBM cards and, using a computer program written for IBM 1620, the total nitrogen for each sample was calculated.

Very low and very high recoveries were conducive to rejection of the data.

E/T ratio: As mentioned in the FAO/WHO 1965 scoring procedure, this ratio was the total amount of essential amino acids in grams per gm nitrogen. The E/T ratios for the proteins of different classes of foodstuffs have already been reported (FAO/WHO, 1965, p. 34). The calculated E/T ratios, on the basis of the data obtained through analysis, were compared with the reported values. The results of these ratios, in conjunction with other check procedures were taken as criteria for accepting or rejecting the runs.

IV. RESULTS AND DISCUSSION

The following section will deal with the discussion of total amino acids, sulfur amino acids, protein quality, and sulfur amino acid sulfur vs. total sulfur in the Middle Eastern foodstuffs and diets.

Total Amino Acids

The amino acid content of 41 samples of Middle Eastern foodstuffs including dairy products, nuts and seeds, legumes, meat products, and two baby food mixtures were determined by the automatic procedure of Spackman <u>et al</u>. (1958). Tryptophan was determined by a chemical procedure (Lombard and De Lange, 1965). Table 3 shows the values in milligrams amino acid per gram nitrogen for 33 of these samples, the rest are shown in Table 3A. As considered previously the sulfur amino acids require special treatment; the values given in Table 3 for cystine are those obtained by the preoxidation procedure, while those for methionine are from normal hydrolysis. The reasons for this will be discussed in detail later. Also included in Table 3 are the eluted ammonia (corrected for ammonia in buffers), the total amino acid nitrogen, three protein quality scores, and the total essential amino acids in gm/gmN (E/T ratio).

The 1957 scores indicate the protein quality value of the samples on the basis of the amount of the most limiting amino acid,

Table 3. Amino acid composition and protein quality scores for selected Middle Eastern foodstuffs (milk, milk products and egg).

Item / Amino acid (mg/gm N)	Casein	Cheeses				Labné	Egg
		Akkawi	Arishé	Moun-tain	Shank-lish		
Tryptophan	83	91	89	74	106	83	96
Threonine	273	222	260	281	266	294	290
Isoleucine	355	347	343	306	359	384	358
Leucine	634	647	656	638	704	723	584
Lysine	524	476	538	522	674	545	455
SAA[x] (Methionine	162	168	210	193	209	197	213
(Cystine	21	33	38	34	46	50	155
(Total	183	201	248	227	255	247	368
Phenylalanine	344	341	357	285	365	379	361
Tyrosine	362	362	358	276	385	395	335
Valine	478	468	506	519	482	504	482
Arginine	254	245	218	211	249	240	454
Histidine	187	201	194	197	196	178	176
Alanine	204	188	215	138	263	261	382
Aspartic acid	471	455	469	387	502	565	626
Glutamic acid	1312	1281	1314	1355	1285	1478	816
Glycine	125	128	125	95	140	151	199
Proline	979	790	780	915	810	800	203
Serine	425	441	311	296	329	392	450
NH_3	137	144	178	167	160	146	126
Total N (gm)	1.02	0.99	1.02	0.98	1.07	1.07	1.00
Score[xx] (FAO 1957	68(S)	74(S)	91(S)	82(Tr)	94(S)	92(S)	107(Tr)
(FAO/WHO 1965	53(S)	59(S)	69(S)	67(S)	66(S)	64(S)	88(Th)
(1000 S/N	65	73	84	77	72	-	121
E/T	3.24	3.16	3.36	3.13	3.60	3.55	3.33

[x] SAA = Sulfur amino acids.

[xx] Letters in brackets denote the limiting amino acids; L = lysine; Tr = tryptophan; S = SAA; Th = threonine.

Table 3. (Continued) - Meat and meat products; Grains and their products.

Amino acid (mg/gm N) \ Item		Lungs (Sheep)	Spleen (Sheep)	Brain (Sheep)	Burghul (Av.2)	Local flour	Arabic bread	Ka'ak
Tryptophan		64	69	60	45	45	38	51
Threonine		245	255	327	172	152	166	157
Isoleucine		198	396	243	195	206	203	220
Leucine		530	556	544	390	400	461	398
Lysine		424	484	359	160	127	133	99
SAA	(Methionine	109	119	155	90	107	99	94
	(Cystine	98	80	96	123	138	138	126
	(Total	207	199	251	213	245	237	220
Phenylalanine		291	284	320	253	293	390	277
Tyrosine		206	182	241	183	202	214	188
Valine		387	408	425	230	271	288	238
Arginine		384	395	337	270	230	264	285
Histidine		171	208	150	120	115	149	130
Alanine		439	404	377	222	173	197	188
Aspartic acid		538	542	665	290	148	274	276
Glutamic acid		719	728	990	1397	1839	2127	1139
Glycine		607	406	348	241	192	209	210
Proline		478	348	381	637	727	834	639
Serine		278	273	428	260	264	239	252
NH_3		123	120	231	197	254	283	247
Total N (gm)		0.97	0.96	1.04	0.85	0.90	1.02	0.84
Score	(FAO 1957	71(Tr)	74(S)	67(Tr)	50(Tr)	47(L)	42(Tr)	37(L)
	(FAO/WHO 1965	75(S)	65(S)	70(Tr)	68(L)	52(L)	50(L)	43(L)
	(1000 S/N	79	72	-	106	109	89	96
E/T		2.55	2.83	2.77	1.89	1.94	2.11	1.85

Table 3. (Continued) - Legumes.

Amino acid (mg/gm N) \ Item	Chick peas	Fenu-greek	Lentils	Soybean (Av.3)	Broad beans (green)	Sweet lupine
Tryptophan	86	45	36	82	33	42
Threonine	248	201	266	255	159	237
Isoleucine	296	298	282	313	222	295
Leucine	501	409	485	537	389	472
Lysine	463	357	484	450	338	304
SAA (Methionine	91	82	60	87	33	41
SAA (Cystine	93	100	60	104	38	97
SAA (Total	184	182	120	191	71	138
Phenylalanine	488	237	327	352	215	252
Tyrosine	212	191	211	273	153	299
Valine	331	237	353	346	374	308
Arginine	537	646	555	514	760	679
Histidine	168	144	200	187	126	138
Alanine	271	225	268	284	255	238
Aspartic acid	751	622	716	757	659	682
Glutamic acid	1028	967	1007	1156	781	1328
Glycine	179	269	262	275	201	270
Proline	330	301	282	408	206	298
Serine	319	279	321	336	240	325
NH_3	125	116	100	123	202	157
Total N (gm)	0.98	0.91	0.95	1.02	0.95	0.95
Score (FAO 1957	68(S)	50(Tr)	40(Tr)	71(S)	26(S)	47(Tr)
Score (FAO/WHO 1965	61(S)	67(Tr)	44(S)	63(S)	34(S)	55(S)
Score (1000 S/N	77	64	56	65	52	58
E/T	2.81	2.16	2.52	2.80	1.95	2.35

Table 3. (Continued) - Legumes; Kishk.

Amino acid (mg/gm N) \ Item	Peanut 1	Peanut 2	Peanut 3	Peanut 4	Peanut 5	Kishk
Tryptophan	56	62	53	53	53	61
Threonine	175	173	207	165	160	205
Isoleucine	198	224	194	212	215	272
Leucine	390	433	402	400	409	512
Lysine	236	238	184	196	208	324
(Methionine	64	57	58	56	56	121
SAA (Cystine	73	55	79	62	77	107
(Total	137	112	137	118	133	228
Phenylalanine	231	317	302	340	330	314
Tyrosine	316	259	280	260	268	261
Valine	241	228	276	299	313	326
Arginine	702	772	682	710	733	313
Histidine	166	172	105	153	139	167
Alanine	195	245	239	263	251	235
Aspartic acid	748	765	648	722	729	375
Glutamic acid	1183	1032	1176	1220	1279	1563
Glycine	383	351	370	380	365	217
Proline	230	309	305	299	294	745
Serine	221	146	285	301	300	299
NH_3	124	142	137	124	146	228
Total N (gm)	0.94	0.97	0.94	0.97	1.00	1.01
(FAO 1957	51(S)	42(S)	51(S)	44(S)	50(S)	68(Tr)
Score (FAO/WHO 1965	64(S)	51(S)	63(S)	54(S)	60(S)	78(Tr)
(1000 S/N	50	49	48	52	51	95
E/T	1.98	2.05	2.04	2.04	2.09	2.50

Table 3. (Continued) - Nuts and seeds; Baby food mixtures.

Amino acid (mg/gm N) \ Item	Dry almond	Green almond	Pistachio Nut	Water-melon seeds	Pine seeds	Laubina 104 (Av.2)	Laubina 105
Tryptophan	35	25	58	73	47	50	42
Threonine	152	179	172	210	122	188	203
Isoleucine	216	177	247	308	179	259	252
Leucine	391	309	427	438	357	468	459
Lysine	140	249	303	162	156	343	256
SAA (Methionine	44	62	103	161	93	100	96
SAA (Cystine	65	64	101	70	138	93	107
SAA (Total	109	126	204	231	231	193	203
Phenylalanine	301	207	305	496	176	308	332
Tyrosine	183	136	187	195	208	213	209
Valine	325	229	377	335	249	314	320
Arginine	610	218	521	962	1082	382	387
Histidine	139	93	132	149	125	158	146
Alanine	242	254	263	313	246	209	247
Aspartic acid	589	1560	541	539	447	464	489
Glutamic acid	1366	435	1245	1092	570	1336	1508
Glycine	326	192	283	340	265	197	135
Proline	303	277	251	271	279	609	570
Serine	219	295	346	277	143	287	287
NH_3	202	295	141	108	91	181	225
Total N (gm)	0.96	0.88	0.92	1.04	0.89	0.94	0.97
Score (FAO 1957	39(Tr)	28(Tr)	64(Tr)	60(L)	52(Tr)	56(Tr)	47(Tr)
Score (FAO/WHO 1965	55(S)	49(Tr)	77(Th)	53(L)	72(Th)	69(Tr)	59(Tr)
Score (1000 S/N	62	74	67	75	91	92	95
E/T	1.85	1.64	2.28	2.45	1.73	2.34	2.28

denoted by letters in parantheses (S, L, Tr, Th, for sulfur amino acids, lysine, tryptophan and threonine, respectively), as compared to the respective value for that amino acid in the hypothetical amino acid pattern of FAO 1957. The 1965 scores also show the protein quality of the samples by comparing the proportion of the most limiting amino acid to the total essential amino acids in a sample with the proportion of the same amino acid to the total essential amino acids in egg. The S/N ratio is the score determined by the sulfur and nitrogen contents of each sample. Scores are normally of value for mixed diets; it is, however, useful to give these values together with the amino acid data since the limiting amino acid is specified and it is easier to visualize its degree of deficiency.

In 23 samples the same amino acid is found to be the most limiting one by both 1957 and 1965 scoring procedures. In cases where there are discrepancies, the second limiting amino acid is very close to the first. The S/N ratios are usually close to either one or the other of the 1957 and 1965 scores, especially when the sulfur amino acids are the most limiting ones. In 19 out of the 33 samples the most limiting amino acids are the sulfur amino acids; 14 of these are confirmed by both 1957 and 1965 scoring procedures and 5 only by the 1965 procedure. The next limiting amino acid is mostly tryptophan, then lysine and finally threonine. The E/T ratios agree rather closely with those reported by the FAO/WHO 1965 report. Low E/T values for green almond, dry almond, pine seeds, and burghul as well as high E/T ratios for egg and milk products are noteworthy.

There seems to be a tendency for bigger deviations between the

results of the two scoring procedures, 1957 and 1965, with increasing values for the E/T ratio. Thus, in general, the biggest discrepancies between the two scores are found among the dairy products and egg.

Among the individual amino acid figures the low tryptophan contents for burghul, sweet lupine, Arabic bread, local flour, lentils, soybean, broad beans, dry and green almonds are striking, in contrast to the relatively high figures for egg and dairy products. A cereal-legume mixture based upon lentils and burghul, or burghul and broad beans, would always be low in tryptophan and a protein source high in tryptophan such as chick peas, soybean, egg or a milk product would need to be incorporated in order to produce a protein of high value. The foodstuffs high in methionine or total sulfur amino acids are of importance because most world diets are limiting in sulfur amino acids (Rose et al., 1954). Other than egg, whose importance as a methionine source is well documented, the cheeses, labné, brain, and watermelon seeds are high in methionine. Good sources of lysine to supplement the lack of lysine in wheat flour products are the milk products, chick peas, lentils, soybean, spleen and kishk.

Table 3A also shows the amino acid content of several foodstuffs in mg/gm N. Tryptophan values have not yet been determined and the sulfur amino acid data were only obtained from unoxidized samples. The table also includes the eluted ammonia, the total amino acid nitrogen and the 1957 scores. The 1965 scores could not be calculated since the tryptophan values were not available. Among the individual figures high levels of lysine in goat laban and green chick peas, also relatively high quantities of methionine in tehineh and goat laban are to be noted.

Table 3A. Amino acid composition and protein quality scores for selected Middle Eastern foodstuffs.

Amino acid (mg/gm N) \ Item	Green chick peas	Persian porenj	Sweetened lupine	Mountain bread (Mar'ouk)
Tryptophan	-	-	-	-
Threonine	195	156	254	162
Isoleucine	235	449	326	197
Leucine	410	884	554	386
Lysine	435	149	260	112
SAA (Methionine	100	97	65	88
SAA (Cystine[x]	70	111	70	99
SAA (Total	170	208	135	187
Phenylalanine	263	336	276	271
Tyrosine	186	213	303	190
Valine	318	282	225	215
Arginine	679	266	700	251
Histidine	191	137	143	114
Alanine	251	210	241	207
Aspartic acid	662	313	724	286
Glutamic acid	830	233	1109	1678
Glycine	231	257	282	227
Proline	307	712	307	626
Serine	275	284	355	238
NH_3	228	247	159	228
Total N (gm)	1.03	0.88	1.01	0.86
FAO 1957 score	63(S)	55(L)	50(S)	41(L)

[x] From ordinary hydrolysis.

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Table 3A. (Continued).

Amino acid (mg/gm N) \ Item	Goat laban	Tehineh	Baby food (Senegal)	Cerelac
Tryptophan	-	-	-	-
Threonine	284	219	206	180
Isoleucine	309	216	272	240
Leucine	618	414	607	489
Lysine	528	166	288	152
(Methionine	159	174	89	107
SAA (Cystine	27	113	67	24
(Total	186	287	156	131
Phenylalanine	275	285	347	257
Tyrosine	257	246	303	221
Valine	488	293	380	184
Arginine	203	819	528	197
Histidine	179	165	169	137
Alanine	200	304	317	161
Aspartic acid	445	518	637	304
Glutamic acid	1294	1216	1373	1486
Glycine	115	315	281	147
Proline	796	241	499	735
Serine	301	294	330	276
NH_3	189	140	152	411
Total N (gm)	0.98	1.00	1.03	0.99
FAO 1957 score	69(S)	61(L)	58(S)	49(S)

Sulfur Amino Acids

As discussed earlier, the sulfur amino acids are amongst the most labile and are also the most likely to be limiting in human dietaries, thus special procedures have been advocated for their analysis. All the samples reported in Table 3 were analyzed by a preoxidation procedure outlined earlier, in addition to the normal direct hydrolytic procedures.

Preoxidation converted cystine to cysteic acid and methionine to methionine sulfone and they were separated and measured in this form by the subsequent ion exchange chromatography. The elution times at 30 ml per hour flow rate for the various sulfur amino acids were approximately 110 minutes for cysteic acid, 230 minutes for methionine sulfoxides, 280 minutes for methionine, 600 minutes for cystine and 690 minutes for methionine. Normally, elution is stopped after the emergence of methionine sulfone whether special elution techniques are used (Bujard and Mauron, 1963; Lewis, 1966) or whether the normal chromatographic procedure is followed. Low recoveries of methionine were noted compared to the values obtained when the normal hydrolytic procedure was followed and it was considered that perhaps some methionine was remaining unoxidized. By eluting the oxidized samples for 12 hours it was observed that there was indeed a small quantity of residual methionine which had resisted oxidation. Had the oxidation procedure been at fault, had there been, for example, insufficient penetration of the oxidizing reagent to the sample, one would have imagined that there would have been residual unoxidized cystine present.

This was not the case, in no oxidized sample was there any free cystine remaining; complete oxidation to cysteic acid always took place. For twenty five of the above samples elution was continued till after the emergence of methionine. Table 4 shows the distribution of the sulfur amino acids obtained for most of these samples. For cystine the values obtained from preoxidized samples came from cysteic acid only, while the cystine from normal hydrolysis was the total of cysteic acid and unchanged cystine. In the case of methionine the preoxidized values were the total of methionine sulfone and unchanged methionine (no methionine sulfoxides). Methionine from normal hydrolysis, however, included methionine sulfoxides and intact methionine (no methionine sulfone).

There was in all cases some methionine left intact but nevertheless the total value obtained after oxidation was still below that obtained with normal hydrolysis. Cystine showed either no change or a net increase due to oxidation.

The mean values obtained for both mixed diets and individual food items are of interest and are found in Table 5. As shown, there was on the average a net increase either in absolute or percentage terms in the cystine content of 31 samples following the preoxidation procedure. However, there was in general a net loss in methionine content for 25 samples both in absolute and percentage values, whether the elution was continued as normal, that is until methionine sulfone was eluted or whether the analysis time was extended for the elution of any residual methionine (about 12 hours). The loss in the latter case, however, was less, due to the presence of a small quantity of

Table 4. Effect of preoxidation on the cystine and methionine content of some Middle Eastern food items.

Sample description	Sulfur amino acids (mg/gm N)							
	Cystine		Methionine					
	Pre-oxidized	Normal hydrolysis	Preoxidized			Normal hydrolysis		
			As meth. sulfone	Methio-nine	Total methio-nine	As meth. sulfoxides	Methio-nine	Total methio-nine
Casein	21	0	171	3	174	25	137	162
Egg	155	172	171	19	190	0	213	213
Akkawi Cheese	33	29	118	9	127	16	152	168
Mountain "	34	16	110	5	115	21	172	193
Arishé "	38	35	116	2	118	30	180	210
Dry almond	65	67	33	1	34	31	13	44
Green almond	64	94	47	9	56	13	49	62
Pistachio nut	101	104	92	2	94	72	31	103
Brain	96	86	116	3	119	47	108	155
Lungs	98	111	106	3	109	38	71	109
Broad beans, fresh	38	30	32	1	33	18	0	18
Soybean	104	90	98	1	99	19	68	87
Peanut	79	57	63	9	72	33	25	58
Wheat flour, local	138	114	110	5	115	29	78	107
Ka'ak	126	87	87	12	99	0	94	94
Kishk	107	99	108	14	122	0	121	121

Table 5. Effect of oxidation and time of elution upon the mean recovery of cystine and methionine.

Amino acid[1]	No. of samples	Type of elution	Average increase or decrease in amino acid content caused by preoxidation	
			Absolute (mg/gm N)[2]	Percentage
Cystine	31	Normal	+13.0 ± 2.3	+17.8
Methionine	25	Normal	-14.5 ± 5.5	- 9.7
Methionine	25	Extended	- 8.0 ± 5.6	- 3.2

[1] Cysteic acid, methionine sulfoxides, and methionine sulfone were converted by calculation to the equivalents of their parent amino acids. For cystine the unoxidized sample was the total of cysteic acid and unchanged cystine; the oxidized sample was completely in the form of cysteic acid. For methionine the unoxidized sample was the total of methionine sulfoxides and unchanged methionine (no methionine sulfone), while the oxidized sample was the total of methionine sulfone and unchanged methionine (no methionine sulfoxides).

[2] ± Standard error of the mean.

residual methionine.

Because of the variability in methionine recovery following oxidation and the time needed to elute the residual unoxidized methionine, the following procedure was adopted for the results of sulfur amino acids shown in Table 3. The oxidation procedure was considered as valid for cystine only, thus the cystine values are those determined after preoxidation. The methionine values are (with one exception) those obtained by normal hydrolysis and the total sulfur amino acids are the sum of the two values. In the case of fresh broad beans the oxidized value was taken for the methionine because it was much higher than that obtained by normal hydrolysis. Furthermore, the value obtained following normal hydrolysis showed an abnormality in that the whole of methionine was present as methionine sulfoxides indicating perhaps that the methionine was linked in the protein in such a way so as to be especially vulnerable to hydrolysis.

Protein Quality of Diets

The quantities of the four potentially limiting amino acids in some Middle Eastern diets are shown in Table 6 together with the E/T ratio (total essential amino acids in gm/gm N) and the total amino acid N recovery. The E/T ratio is a valuable index of quality, thus diet No. 6 with the highest E/T ratio has the best quality as determined biologically (Pellett and Jamalian, 1968). The amino acid figures were used in calculating the protein quality scores on the basis of FAO 1957 or FAO/WHO 1965 pattern. Both the level of the actual limiting amino acid and the level of sulfur amino acids were

Table 6. The level of potentially limiting amino acids in some Middle Eastern diets.

No.	Major constituents of diets	Total E.A.A. (gm/gm N)	Amino acids (mg/gm N)				Total N (gm)
			Try	Thr	Lys	SAA	
1	Bread, rice, lentils	2.30	43	173	229	181	0.98
2	Bread, rice, cheese, yoghurt	2.57	58	185	192	217	0.96
3	Bread, cheese, rice, lentils	2.20	50	195	158	190	0.92
4	Bread, rice, milk, dates	2.11	50	181	204	206	0.94
5	Bread, meat, milk, rice	2.07	55	220	360	185	0.95
6	Bread, cheese, yoghurt, meat, egg	2.72	66	246	449	218	1.09
7	Ring of bread, sesame seeds, za'atar	1.85	51	157	99	220	0.84
8	Burghul, labné with meat, oil, garlic	2.11	61	184	262	183	1.01
9	Arabic bread, meat, tehineh	2.12	47	239	323	195	0.91

used for the calculation since it is generally agreed that the sulfur amino acids are limiting in most human diets and there is also some controversy over the recommended levels of tryptophan in the reference patterns. In addition , the diets were also scored on the basis of their S/N ratios (Miller and Donoso, 1963; Pellett and Eddy, 1964). These scores, obtained in five different ways, were used to calculate the NDpCal percent using the formula of Miller and Payne (1961a) as mentioned earlier. Table 7 compares the results obtained from these different ways. As will be noticed there is little correlation among the five scoring procedures. Variable NDpCal percent values are obtained for the same diet. These procedures do not even clearly show the same trend of increase or decrease in quality of the diets. Comparison of the calculated values for these same diets with the biologically determined ones has been discussed elsewhere (Pellett and Jamalian, 1968). The 1957 procedure using sulfur amino acids gave a reasonably good correlation except for diet 6.7 the protein of which was known to have been damaged. Also S/N ratio showed a good correlation with the biologically determined values for high protein quality diets but failed where lower quality protein was involved.

Sulfur Amino Acid Sulfur vs. Total Sulfur

Reference was made earlier to the importance of sulfur amino acids in determining the quality of mixed diets, since it is generally believed that most human diets are limiting in sulfur amino acids (Rose et al. 1954; Miller and Donoso, 1963). It was also pointed out that the sulfur content of mixed diets could be taken as a more

Table 7. The comparison of NDpCal percent of some Middle Eastern diets calculated by five scoring procedures.

No.[x]	PCal percent	NDpCal percent				
		Using "1957 score"		Using "1965 score"		S/N
		Limiting[xx]	SAA	Limiting[xx]	SAA	
6.1	12.1	5.3(T)	7.1	6.4(T)	7.6	8.5
6.2	11.8	6.6(T)	8.1	6.3(L)	7.9	8.8
6.3	12.3	6.1(T)	7.4	6.2(L)	8.4	9.0
6.4	12.2	6.1(T)	8.0	8.0(T)	9.3	9.4
6.5	18.0	8.3(T)	9.2	10.9(S)	10.9	11.0
6.6	20.9	10.4(T)	11.3	10.7(S)	10.7	11.5
6.7	14.5	4.9(L)	9.4	5.5(L)	11.5	11.0
6.8	9.0	5.8(T)	5.8	6.6(S)	6.6	8.1
6.9	21.8	7.9(T)	10.4	10.3(T)	12.1	11.8

[x] Numbers refer to diets described in Table 6.

[xx] Limiting amino acid; T = tryptophan; L = lysine; S = total sulfur amino acids.

reliable index of protein quality than the level of nitrogen (Miller and Naismith, 1958); this led Miller and Donoso (1963) to propose S/N ratio as a direct quality index score. The ratio was used for British hospital meals by Pellett and Eddy (1964) who showed good correlation between the values predicted from S/N ratio and the biologically determined values.

The fact that protein values could be predicted from analytical data for total sulfur implied not only that the diets were limited by sulfur amino acids but also that there was a constant ratio between the sulfur contributed by the sulfur amino acids and the total sulfur in the diets. To test this hypothesis the ratio was calculated for a number of Middle Eastern foodstuffs and mixed diets as follows:

$$\frac{\text{mg sulfur (from sulfur amino acids) per gm N}}{\text{mg total sulfur per gm N}} \times 100$$

The results for foodstuffs are shown in Table 8 and those for meals and diets in Table 8A. It will be seen that the ratio appeared to be reasonably constant for mixed diets. It was of interest to find that there was also some constancy among the ratios calculated for groups of foodstuffs, though with a higher degree of variability.

The prediction of protein quality from total sulfur is only recommended for mixed diets, the reasonable constancy of the ratio for mixed diets, Table 8A, and its greater variability amongst individual foodstuffs, Table 8, gives some justification to this recommendation. The whole concept of interrelationship between the S/N ratio and sulfur amino acid sulfur and its value in predicting the protein quality will be discussed in detail elsewhere (Pellett <u>et al</u>., manuscript in preparation).

Table 8. Amount of sulfur from sulfur amino acids as percentage of total sulfur in some Middle Eastern foodstuffs.

Milk products:				
	Casein	62.2	Arishé cheese	65.6
	Akkawi cheese	61.8	Mountain "	65.7
	Shanklish "	79.1	Average ± S.E.:	66.9 ± 3.2
Wheat and wheat products:				
	Burghul	48.8	Arabic bread	65.2
	Flour, local	54.8	Ka'ak	56.0
			Average ± S.E.:	56.2 ± 3.4
Legumes:				
	Chick peas	57.6	Peanut (1)	66.9
	Fenugreek	69.3	Peanut (2)	55.2
	Lentils	51.5	Peanut (3)	69.9
	Soybean	71.7	Peanut (4)	54.6
	Sweet lupine	59.1	Peanut (5)	64.4
			Average ± S.E.:	62.0 ± 2.3
Nuts and seeds:				
	Dry almond	42.8	Watermelon seeds	70.4
	Green almond	40.8	Pine seeds	61.8
	Pistachio nut	72.9	Average ± S.E.:	57.7 ± 6.9

Table 8A. Amount of sulfur from sulfur amino acids as percentage of total sulfur in some Middle Eastern meals and diets.

Arabic bread, meat, tehineh ("Shawarma")	56.1
Bread, cheese, yoghurt, meat, egg (rich city diet)	62.1
Bread, meat, milk, rice (Northern-1)	52.5
Bread, rice, lentils (Northern-2)	52.0
Bread, cheese, rice, lentils (poor village-1)	52.2
Bread, rice, milk, dates (poor village-3)	53.8
Low meat, legume	38.1
Egg, milk, kubbé, no legume	38.7
Meat, wheat, cereal, no milk, no legume	68.7
Wheat, milk, legume	57.3
Kubbé	51.3
Meat, fish	47.5
Kishk (burghul, labné, meat, oil, garlic)	56.3
Laubina 104	50.3
Laubina 105	51.5
Average ± S.E.	52.6 ± 2.0

V. SUMMARY AND CONCLUSIONS

1. An automatic amino acid analyzer based on the column chromatographic procedure was used to determine the amino acid composition of 41 items of typical Middle Eastern foodstuffs.

2. Data are presented on the levels of the seventeen amino acids present in acid hydrolyzates of such foodstuffs. The tryptophan values are also presented; these were obtained upon samples hydrolyzed enzymatically.

3. Sulfur amino acids are liable to destruction upon simple acid hydrolysis, thus for their analysis a preoxidation procedure was used followed by acid hydrolysis and subsequent chromatography.

4. Comparison was made between the results for sulfur amino acids obtained through ordinary hydrolysis and those determined by preoxidation. The preoxidation procedure was found to be valid for cystine and not valid for methionine.

5. Protein quality scores were calculated according to the following procedures:

   a. FAO 1957 scoring system.

   b. FAO/WHO 1965 scoring system (used where tryptophan data were available).

   c. S/N ratio.

The S/N ratios were usually close to either one or the other of the 1957 and 1965 scores, especially when the sulfur amino acids were the most limiting.

In 24 out of the 41 samples analyzed, sulfur amino acids were the most limiting according to one or more of the scoring procedures. The remaining 17 were mostly limited by tryptophan, some by lysine, and a few by threonine.

6. NDp Cal percents of a number of Middle Eastern diets were calculated using the three scoring procedures. There was very little correlation between the values obtained by these different procedures; comparison of these values with those determined biologically indicated that no one procedure could be considered as superior to any other as a predictor of protein quality.

7. The ratio of the sulfur amino acid sulfur to total sulfur was calculated for several groups of foodstuffs and a number of mixed diets. The constancy of this ratio for mixed diets and its variability among different groups of foodstuffs confirmed that the S/N ratio could not be used as a predictor of protein quality for individual foodstuffs but that it may have some validity for certain mixed diets.

LITERATURE CITED

Barnes, R.H. 1963. The contributions of Hamilton, Mitchell, and Rose. In Symposium on Protein Nutrition and Metabolism. Editors: Kastellic, J., H.H. Draper, and H.P. Broquist. Special Public. No. 4. University of Illinois. College of Agriculture, Urbana, Illinois.

Benson, J.V. Jr., and J.A. Patterson. 1965a. Accelerated automatic chromatographic analysis of amino acids on a spherical resin. Anal. Chem. 37: 1108.

Benson, J.V., Jr., and J.A. Patterson. 1965b. Accelerated chromatographic analysis of amino acids commonly found in physiological fluids on a spherical resin of specific design. Anal. Biochem. 13: 265.

Bidmead, D., and F.J. Ley. 1958. Quantitative amino acid analysis of food proteins by means of a single ion exchange column. Biochim. Biophys. Acta, 29: 562.

Block, R.J., and D. Bolling. 1951. The Amino Acid Composition of Proteins and Foods. 2nd ed. Thomas, C.C., Springfield, Illinois.

Block, R.J., E.L. Durrum, and G. Zweig. 1958. A Manual of Paper Chromatography and Paper Electrophoresis. Academic Press, New York.

Block, R.J., and K.W. Weiss. 1956. Amino Acid Handbook. Charles C. Thomas, Springfield, Illinois, p. 18.

Bujard, E., and J. Mauron. 1963. Cited by Pellett, P.L. (Editor) in Evaluation of Protein Quality. National Academy of Sciences, National Research Council, Public. No. 1100. Washington, D.C.

Campbell, J.A. 1963. Methodology of Protein Evaluation. Division of Food Technology and Nutrition, Faculty of Agricultural Sciences, American University of Beirut. Beirut, Lebanon, Public. No. 21.

Consden, R., A.H. Gordon, and A.J.P. Martin. 1944. Quantitative analysis of proteins: a partition chromatographic method using paper. Biochem. J. 38: 224.

Corfield, M.C., and A. Robson. 1962. Automatic analysis of amino acids by polarographic estimation of their copper complexes. Biochem. J. 84: 146.

De Belsunce, C., and R. Pion. 1963. Estimation of cystine in foodstuffs. Ann. Biol. Animale Biochim. Biophys. 3: 191.

Drèze, A. 1956. Stability of tryptophan during the alkaline hydrolysis of protein. Biochem. J. 62: 3p.

Dus, K., S. Lindroth, R. Pabst, and R.M. Smith. 1966. Continuous amino acid analysis: elution programming and automatic column selection by means of a rotating value. Anal. Biochem. 14: 41.

Dustin, J.P., E. Schram, S. Moore, and E.J. Bigwood. 1953. Cited by Gorden, W.G. in J.A.O.A.C. 45: 785, 1962.

Eastoe, J.E. 1961. A semi-micro method for the determination of amino acids by ion-exchange chromatography. Biochem. J. 79: 652.

Evans, R.J., and S.L. Bandemer. 1967. Nutritive value of legume seed proteins. J. Agr. Food Chem. 15: 439.

Eveleigh, J.W., H.J. Adler, A.S. Reichler. 1967. A completely automated amino acid analysis system. Page 26 of Technicon European Symposium Abstracts on Automation in Analytical Chemistry. 13th - 15th Nov. 1967, Brighton, England.

FAO. 1957. Protein Requirements. FAO Nutritional Studies, No. 16, Rome.

FAO. 1963a. Amino Acid Content of Foods. Nutrition Division, FAO, Rome.

FAO. 1963b. Third World Food Survey. Food and Agriculture Organization of the United Nations, Rome.

FAO. 1964. Protein: At the Heart of the World Food Problem. Food and Agriculture Organization of the United Nations, Rome.

FAO/WHO. 1965. Protein Requirements. FAO Nutrition Meetings Report Series No. 37/WHO Technical Report Series, No. 301, FAO, Rome.

Finlayson, A.J. 1965. Amino acid recoveries in the analyses of some feed samples. Canadian J. Plant Science, 45: 184.

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Gordon, W.G., and J.J. Basch. 1964. Hydrolysis of proteins for amino acid analysis. II. Acid hydrolysis in sealed tubes of mixtures of β-lactoglobulin and starch. A.O.A.C. 47: 745.

Hamilton, P.B. 1958. Ion exchange chromatography of amino acids. Effect of resin particle size on column performance. Anal. Chem. 30: 914.

Hamilton, P.B. 1963. Ion exchange chromatography of amino acids. A single column, high resolving, fully automatic procedure. Anal. Chem. 35: 2055.

Hamilton, P.B., D.C. Bogue, and R.A. Anderson. 1960. Ion exchange chromatography of amino acid. Anal. Chem. 32: 1782.

Hill, R.L. 1965. Hydrolysis of proteins in Advances in Protein Chemistry. Edited by Anfinsen, C.B., M.L. Anson, J.T. Edsall, and F.M. Richards. Academic Press, N.Y. Vol. 20, p. 37.

Hirs, C.H.W. 1956. The oxidation of ribonuclease with performic acid. J. Biol. Chem. 219: 611.

Horn, M.J., and D.B. Jones. 1945. A rapid colorimetric method for the determination of tryptophane in proteins and foods. J. Biol. Chem. 157: 153.

Jacobs, S. 1966. Determination of amino acids by ion exchange chromatography. In Methods of Biochemical Analysis. Ed. Glick, D., Intersc. Public. XIV.

Karrer, P. 1947. Organic Chemistry. Third English Edition, translated by Mee, A.J., Elsevier Publishing Co., Inc. New York, Amsterdam, London, Brussels.

Kimmel, J.R., H. Markowitz, and D.M. Brown. 1959. Some chemical and physiological properties of erythro cuprein. J. Biol. Chem. 234: 46.

Kofrányi, E. 1964. Estimation of the biological values of food proteins. 6. Estimation of tryptophan in foodstuffs. Hoppe-Seyler's Ztschr. 335: 161 in Nutr. Abst. and Rev. 35: No. 2, Abs. No. 1716.

Kominz, D.R. 1962. A method for the improved resolution of basic amino acids on the automatic amino acid analyzer. J. Chromatography. 9: 253.

Krampitz, G. 1960. Comparative studies on protein hydrolysis with a view to reducing the distruction of the components of the protein. 1. Quantitative estimation of amino acids in protein hydrolysates with the aid of an automatic recording apparatus. 2. Behaviour of free amino acids in conditions of protein hydrolysis. 3. Hydrolysis of pure proteins. Ztschr. Tierphysiol. Tiererenahrung Futtermittelk. 15: 40-46; 76-86; 87-95. In Nutr. Abst. Rev. 30: No. 4, 1193.

Krampitz, G., and J. Wieneke. 1963. Estimation of amino acids in biological material. 7. An automatic apparatus for routine estimation of amino acids. Ztschr. Tierphysiol. Tiererenahrung Futter-mittelk. 18: 147. In Nutr. Abs. & Rev. 34: No. 2, Abs. No. 1911.

Lederer, E., and M. Lederer. 1957. Chromatography. 2nd Ed. Elsevier Publishing Co., Inc., New York.

Lewis, O.A.M. 1966. Short ion exchange column method for the estimation of cystine and methionine. Nature, 209: 1239.

Lombard, J.H., and D.J. De Lange. 1965. The chemical determination of tryptophan in foods and mixed diets. Anal. Biochem. 10: 260.

Lyman, C.M., B.J. Camp, and H.M. Tiller. 1958. The tyrosine content of farm feeds. J. Agr. Food Chem. 6: 767.

Macpherson, H.T. 1946. The basic amino acid content of proteins. Biochem. J. 40: 470-480.

Martin, A.J.P., and R.L.M. Synge. 1941. Some applications of periodic acid to the study of the hydroxy-amino-acids of protein hydrolysates. Biochem. J. 35: 294.

Meister, A. 1965. Biochemistry of the Amino Acids. 2nd Ed. Vol. I, Academic Press, N.Y.

Meister, A. 1965. Biochemistry of the Amino Acids. 2nd Ed. Vol. II, Academic Press, N.Y.

Miller, D.S., and G. Donoso. 1963. Relationship between the Sulphur/Nitrogen ratio and the protein value of diets. J. Sci. Fd. Agric. 14: 345.

Miller, D.S., and D.J. Naismith. 1958. A correlation between sulphur content and net dietary-protein value. Nature. 182: 1786.

Miller, D.S., and P.R. Payne. 1959. A ballistic bomb calorimeter. Brit. J. Nutr. 13: 501.

Miller, D.S., and P.R. Payne. 1961a. Problems in the prediction of protein values of diets: The influence of protein concentration. Brit. J. Nutr. 15: 11.

Miller, D.S., and P.R. Payne. 1961b. Problems in the prediction of protein values of diets: The use of food composition tables. J. Nutrition. 74: 413.

Miller, D.S., and P.R. Payne. 1961c. Problems in the prediction of protein values of diets: Caloric restriction. J. Nutrition, 75: 225.

Mondino, A. 1967. A new system of automatic amino acid analysis. J. Chromatography, 30: 100.

Moore, S. 1963. On the determination of cystine as cysteic acid. J. Biol. Chem. 238: 235.

Moore, S., D.H. Spackman, and W.H. Stein. 1958. Chromatography of amino acids on sulfonated polystyrene resins. Anal. Chem. 30: 1185.

Moore, S., and W.H. Stein. 1948. Photometric ninhydrin method for use in the chromatography of amino acids. J. Biol. Chem. 176: 367.

Moore, S., and W.H. Stein. 1951. Chromatography of amino acids on sulfonated polystyrene resins. J. Biol. Chem. 192: 663.

Moore, S., and W.H. Stein. 1954. Procedures for the chromatographic determination of amino acids on four percent cross-linked sulfonated polystyrene resins. J. Biol. Chem. 211: 893.

Orr, M.L., and B.K. Watt. 1957. Amino Acid Content of Foods. Home Economics Research Report No. 4, United States Department of Agriculture.

Payne-Botha, S., and E.J. Bigwood. 1959. Amino acid content of raw and heat-sterilized cow's milk. Brit. J. Nutr. 13: 385.

Payne, P.R., D.S. Miller, and B.S. Platt. 1961. The use of a desiccant in the freeze-drying of foods on a laboratory scale. Proc. Nutr. Soc. 20: XII.

Pellett, P.L. (Editor). 1963. Evaluation of Protein Quality. National Academy of Sciences, National Research Council, Public. No. 1100, Washington, D.C.

Pellett, P.L. 1965. Urinary sulphate sulphur as a measure of the protein value of diets. Proc. Nutr. Soc. 24: XXXVII.

Pellett, P.L., and T.P. Eddy. 1964. The protein-calorie value of British hospital meals. Brit. J. Nutr. 18: 567.

Pellett, P.L., and J. Jamalian. 1966. Investigation of net dietary protein value of the Middle Eastern dietaries. Page 27 in Proceedings of the Second Symposium on Human Nutrition and Health in the Near East, Beirut, Lebanon.

Pellett, P.L., and J. Jamalian. 1968. Observations on the protein-calorie value of Middle Eastern foods and diets. Proceedings of the Nutrition Symposium, A.U.B., in the process of publication.

Pellett, P.L., A. Kantarjian, and J. Jamalian. 1968. The use of sulfur analysis in the prediction of protein-calorie value of Middle Eastern diets. (Manuscript in preparation).

Piez, K.A., and L. Morris. 1960. A modified procedure for automatic analysis of amino acids. Anal. Biochem. 1: 187.

Platt, B.S., D.S. Miller, and P.R. Payne. 1961. Protein values of human food. Recent Advances in Clinical Nutrition. Ed. J.F. Brock. p. 351.

Rees, M.W. 1946. The estimation of threonine and serine in proteins. Biochem. J. 40: 632.

Riesen, W.H., B.S. Schweigert, and C.A. Elvehjem. 1946. Microbiological determination of methionine in proteins and foodstuffs. J. Biol. Chem. 165: 347.

Rinetti, M., and L. Traineas. 1961. Automatic amino acid analysis. Minerva dietol. 1: 96 in Nutr. Abs. and Rev. 32: No. 2, Abs. No. 1687.

Rose, W.C., W.J. Haines, and D.T. Warner. 1954. The amino acid requirements of man. J. Biol. Chem. 206: 421.

Sanger, F. 1949. Fractionation of oxidized insulin. Biochem. J. 44: 126.

Schram, E., J.P. Dustin, S. Moore, and E.J. Bigwood. 1953. Application of ion exchange chromatography to the study of the amino acid content of foods. Anal. Chim. Acta, 9: 149.

Schram, E., S. Moore, and E.J. Bigwood. 1954. Chromatographic determination of cystine as cysteic acid. Biochem. J. 57: 33.

Schweigert, B.S., J.M. McIntire, C.A. Elvehjem, and F.M. Strong. 1964. The direct determination of valine and leucine in fresh animal tissues. J. Biol. Chem. 155: 183.

Scott, T.A. 1961. An enzymic method for the estimation of L-tryptophan. Biochem. J. 80: 462.

Sebeck, O.K. 1965. Microbiological method for the determination of L-tryptophan. J. Bacteriol. 90: 1026.

Simmonds, D.H., and R.J. Rowlands. 1960. Automatic equipment for simultaneous determination of amino acids separated on several ion exchange resin columns. Anal. Chem. 32: 259.

Sleim, A.S.M., and N.N. Messiha. 1966. A simple chromatographic method for determining the basic amino acids in protein hydrolyzates. Analyst. 91: 261-267. In Nutr. Abs. and Rev. 36: No. 4. Abs. No. 5639.

Smith, E.L., and A. Stockwell. 1954. Amino acid composition of crystalline carboxypeptidase. J. Biol. Chem. 207: 501.

Smith, P., Jr., M.E. Ambrose, and G.M. Knobl, Jr. 1965. Possible interference of fats, carbohydrates, and salts in amino acid determinations of fish meals and fish protein concentrates and mixed animal diets. J. Agr. Food Chem. 13: 266.

Spackman, D.H., W.H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. 30: 1190.

Spies, J.R. 1950. Determination of tryptophan with p-dimethylaminobenzaldehyde. Anal. Chem. 22: 1447.

Spies, J.R., and D.C. Chambers. 1948. Chemical determination of tryptophan. Anal. Chem. 20: 30.

Spies, J.R., and D.C. Chambers. 1949. Chemical determination of tryptophan in proteins. Anal. Chem. 21: 1249.

Spies, J.R., and D.C. Chambers. 1950. Determination of tryptophan with p-dimethylaminobenzaldehyde using photochemical development of color. Anal. Chem. 22: 1209.

Stefanye, D., and L. Spero. 1964. Automatic amino acid analysis of replicate samples. Anal. Biochem. 8: 265.

Stein, W.H., and S. Moore. 1948. Chromatography of amino acids on starch columns. Separation of phenylalanine, leucine, isoleucine, methionine, tyrosine, and valine. J. Biol. Chem. 176: 337.

Tkachuk, R. 1966. Amino acid composition of wheat flours. Cereal Chem. 43: 207.

Toennies, G. 1942. The oxidative conversion of casein into protein free of methionine and tryptophane. J. Biol. Chem. 145: 667..

Triebold, H.O., and L.W. Aurand. 1963. Food Composition and Analysis. D. Van Nostrand Co., Inc., N.Y.

Tristram, G.R. 1946. Observations upon the application of partition chromatography to the determination of the mono-amino-acids in proteins. Biochem. J. 40: 721.

Tristram, G.R. 1949. Amino acid composition of purified proteins. In Advances in Protein Chemistry. Edited by Anson, M.L., and J.T. Edsall. Academic Press, N.Y. Vol. 5, p. 83.

Tristram, G.R., and R.H. Smith. 1963. The amino acid composition of some purified proteins. In Advances in Protein Chemistry. Edited by Anfinsen, C.B., Academic Press, N.Y. Vol. 18, p. 227.

Wahlroos, Ö, and H. Land. 1959. Separation on a preparative scale of amino acids from a protein hydrolyzate. Acta Chem. Scand. 13: 595.

Wapnir, R.A., and S.P. Bessman. 1965. Assay of free tryptophan in urine by high-voltage electrophoresis. Anal. Biochem. 12: 516.

APPENDICES

APPENDIX A

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Sulfur Amino Acids

"As indicated in the NAC-NRC Public. No. 1100, it is quite possible to obtain a convenient separation with the procedure described therein ....

<u>Oxidation of the sample:</u> Performic acid reagent (for 3 samples), according to Bidmead and Ley (Biochem. Biophys. Acta. 29: 562-567, 1958) but with a reagent twofold concentrated in hydrogen peroxide.

72 ml formic acid, 98 to 100 percent
1.5 ml methanol (to prevent the reagent from freezing)
7.5 ml H_2O_2, 30 percent.

The mixture is kept for two hr. at rm. temp. in a closed vessel to allow the maximum formation of performic acid.

<u>Preparation of the sample:</u> In a round-bottom flask with a rodage weigh a sample corresponding to 7 mg of total Kjeldahl nitrogen and suspend it in some drops of performic acid.

The sample and the performic reagent are cooled at -10^{0} to -14^{0}C for ½ hr. Then 25 ml of the oxidative agent are added to the sample and left overnight (15 hr.) at 4^{0}C. Dilute the mixture with an equal volume of cold distilled H_2O, add cautiously 2 ml HBr, 48 percent (cf. Moore - J. Biol. Chem. <u>238</u>: 235, 1963) and concentrate in an evaporator (at 35^{0}C) after having added about 20 ml of 1N-NaOH to the condenser to absorb the bromine which distils over. The HBr, a reducing agent, destroys the excess of performic acid before hydrolysis of oxidized proteins and can thus increase the precision of the measurement of cysteic acid and methionine sulfone.

Rinse the dried residue well with H_2O, then hydrolyze for 18 hr. with 100 ml 6N HCl with reflux.

Concentrate again, wash well with H_2O and dilute the residue with 20 ml sample dilutor buffer pH 2.2.

2 ml are taken for the chromatography".

E. Bujard

APPENDIX B

THE PHOENIX STANDARD AMINO ACID CALIBRATION TEST MIXTURE #K-18[x]

Amino Acid	uM/ml
DL-aspartic acid	2.50 ± .05 uM
DL-threonine	2.50 ± .05 uM
DL-serine	2.50 ± .05 uM
L-glutamic acid	2.50 ± .05 uM
L-proline	2.50 ± .05 uM
Glycine	2.50 ± .05 uM
DL-alanine	2.50 ± .05 uM
L-cystine	1.25 ± .05 uM
DL-valine	2.50 ± .05 uM
DL-methionine	2.50 ± .05 uM
L-isoleucine	2.50 ± .05 uM
L-leucine	2.50 ± .05 uM
L-tyrosine	2.50 ± .05 uM
DL-phenylalanine	2.50 ± .05 uM
L-lysine	2.50 ± .05 uM
L-histidine	2.50 ± .05 uM
Ammonium sulfate	2.50 ± .05 uM (as NH_3)
L-arginine	2.50 ± .05 uM

[x] Amino acids in a solution of 0.1N HCl.

NOTE: The standard test mixture is to be diluted 1 ml to 5 ml (1 ml standard test mixture added to 4 ml of buffer) with 0.2N, pH 2.2 sodium citrate buffer to give 0.5 micromoles per ml, for application on the ion exchange column.

APPENDIX C

Purification of Nitrogen Gas

It was found, at the early stages of the study, that the total N recovered in the hydrolyzate was more than the N contributed by the sample. It was suspected that perhaps the N_2 gas which was flowing from a cylinder during hydrolysis was not pure, thus the following simple procedure was used to purify the gas from any traces of ammonia. The slow stream of nitrogen gas from the cylinder was passed first through a solution of 4N HCl with phenol red indicator (a few drops) and next through distilled water. The acid picked up any ammonia impurity flowing along with the N_2 gas and the distilled water washed the gas from any acid fumes passing over. A piece of glass wool was put in the acid solution to help break the big N_2 bubbles into smaller more continuous ones. The indicator showed when the acid had been neutralized by the ammonia and thus had to be replaced by a fresh batch.

APPENDIX D

Preparation and Installation of Reagents[1]

Sodium citrate buffers:

The Moore and Stein analytical method, requires four different sodium citrate buffers as eluents. These buffers must be prepared in advance using deionized or distilled water of high purity and are stored for subsequent use. In addition, a small amount of pH 2.2 sodium citrate buffer is prepared and stored for use in diluting and adjusting pH of the samples to be analyzed. A similarly small quantity of pH 3.25 filling buffer is prepared for filling the chromatographic tubes after samples have been applied to the column. Both of these latter two buffers have high thiodiglycol (TG) concentrations which help to minimize the conversion of small amounts of methionine to the methionine sulfoxides during the application of the sample to the column. The use of octanoic acid as a preservative permits these buffers to be completely prepared, after which these buffers can be stored at room temperature.

BRIJ-35 detergent solution:

A storable quantity of BRIJ-35 solution should be prepared for later use in the compounding of the buffers, (50 gm of BRIJ-35 dissolved in 100 ml of hot de-ionized water).

Preparation of buffers:

To prepare a sodium citrate buffer citric acid and NaOH are weighed and dissolved in water in a volumetric flask. HCl, octanoic acid, TG and BRIJ-35 are added and the flask is cooled in a container of cold water, after which the flask is filled to volume. The concentrated buffer solution is poured into the carboy, where it is mixed thoroughly. The pH of the buffer is checked and adjusted by the addition of 50 percent NaOH or concentrated HCl.

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[1] From Phoenix Instruction Manual, Phoenix Precision Instrument Company, Philadelphia 40, Pensylvania.

Transfer to reservoirs in analyzer:

The buffer is filtered and transferred from the carboy to the instrument buffer reservoir.

Regeneration reagents:

0.2N NaOH is used for stripping the basic amino acids from the columns after the conclusion of the analysis for the acidic and neutral components. Deaerated pH 3.25 buffer is used to equilibrate the long columns after the NaOH wash. The 0.2N NaOH reagent is prepared by adding BRIJ-35 detergent solution to 0.2N NaOH.

Ninhydrin reagent:

In addition to ninhydrin, stannous chloride used as the reducing agent, is dissolved in a solvent which is 75 percent methyl cellosolve and 25 percent 4N sodium acetate buffer. Each new lot of methyl cellosolve to be used in the preparation of the reagent should be checked for peroxide content since peroxides destroy the reducing power of the stannous chloride.

To test for the presence of peroxides, 3 ml of the methyl cellosolve is mixed with 3 ml of a 4 percent aqueous solution of potassium iodide. A yellow color indicates the presence of peroxides and a colorless solution indicates their absence.

Sodium acetate buffer:

To 8 liters of de-ionized (or distilled) water, 10,880 gm of $NaOAc.3H_2O$ (reagent grade) is added in a bottle which can be filled accurately to 20 liters. The mixture is stirred in a steam or water bath until solution is complete. Then the solution is cooled to room temperature and 2000 ml of glacial acetic acid is added and the volume of the solution is brought to 20 liters by distilled water.

Preparation of ninhydrin reagent:

3 liters of filtered, peroxide free methyl cellosolve is added to the reagent transfer bottle. Nitrogen is bubbled into the solution and it is stirred magnetically for 15 minutes. Then 1 liter of the prepared and filtered 4N sodium acetate buffer is added, after which the mixture is stirred magnetically for 20 minutes maintaining the N_2 bubbling. Then 80.0 gm of ninhydrin is added to the bottle and the mixture is stirred magnetically, maintaining the N_2 bubbling for 20 minutes. When all the ninhydrin is dissolved, the stirring and nitrogen bubbling is stopped and 1.50 gm of stannous chloride. $SnCl_2.2H_2O$, is added and is washed down into the solution. Then it is stirred magnetically, maintaining N_2 bubbling for 20 minutes. The reagent is then ready for use and is transferred to the ninhydrin reservoir under nitrogen pressure.

Sodium citrate buffers:

pH Use	2.2 ± 0.03 Sample Dilutor	3.25 ± 0.01 Column 1, 1A Eluent	4.25 ± 0.02 Column 1, 1A Eluent	4.26 ± 0.02 Column 2A Eluent	5.28 ± 0.02 Column 2 Eluent	3.25 ± 0.01 Filling Buffer
Sodium Concentration	0.20 N	0.20 N	0.20 N	0.38 N	0.35 N	0.20 N
Citric Acid .H_2O	21 gm	840 gm	840 gm	532 gm	491 gm	21 gm
NaOH (97 percent)	8.4 gm	330 gm	330 gm	312 gm	288 gm	8.3 gm
Concentrated HCl	16 ml	426 ml	188 ml	307 ml	136 ml	10.6 ml
Octanoic Acid	0.1 ml	4.0 ml	4.0 ml	2.0 ml	2.0 ml	0.1 ml
Thiodiglycol (TG)	20 ml	200 ml	200 ml	-	-	20 ml
BRIJ - 35 Solution	2 ml	80 ml	80 ml	40 ml	40 ml	2 ml
Final Volume	1 liter	40 liters	40 liters	20 liters	20 liters	1 liter

APPENDIX E

Automatic Amino Acid Analysis[2]

History

For more than a decade in the Biochemistry Laboratories of the Rockefeller Institute for Medical Research, a concerted effort has been exerted toward devising rapid and reliable ion exchange chromatographic techniques for quantitative evaluation of the amino acid compositions of proteins, physiological fluids and tissues. The development of chemical methods and instrumentation by Dr. Darrel H. Spackman, Dr. William H. Stein, and Dr. Stanford Moore led to automatic equipment for ion exchange separation and continuous photometric amino acid analysis with ninhydrin as described in Analytical Chemistry 30, No. 7, pages 1190-1206 (1958).

Their apparatus was subsequently incorporated into a suitable unit design developed by Dr. Kenneth R. Woods of the Laboratory of Applied Protein Chemistry, Dept. of Medicine, Cornell University Medical College, New York. The Phoenix Model K-8000 Amino Acid Analyzer is the latest improved commercial version of the instruments referred to above.

General description

The instrument comprises a complete, self-contained system for automatic separation, identification, and quantitative analysis of amino acids and other ninhydrin-reactive compounds in unknown mixtures.

Separation is achieved by rapid elution of samples through cation exchange resins. Because of characteristic affinities of amino acids for the resin, the compounds are differentially retarded, separated and eluted in sequence. Only three buffers and 22 hours of automatic unattended operation are required to analyze a protein hydrolyzate, or 40 hours for more complex mixtures with the standard instrument.

Identification of compounds is possible by virtue of characteristic volumes of eluting solution required to displace them from the resin columns. Accurate metering by micro pumps assures volume delivery proportional with elapsed time.

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[2] From Phoenix Instruction Manual, Phoenix Precision Instrument Company, Philadelphia 40, Pensylvania.

<u>Quantitation</u> of each compound is provided by addition of ninhydrin reagent to the steam of eluent from the ion exchange columns. The mixture then passes through a reaction vessel maintained at 100 degrees C, where the ninhydrin reacts with amino acids to produce colored compounds. The absorbance values of the colored compounds are evaluated by continuous recording photometry. A rapid and simple method of integration of peak areas inscribed by the recorder, provides data which can be converted directly to micromoles or milligrams of amino acid.

<u>Operation</u>

Under present operating conditions, a complete automatic analysis of a protein or peptide hydrolyzate may be carried out each day, requiring approximately 22 hours of actual instrument time. The Model K-8000 is designed to permit a sequence of operations such that all procedures connected with performing the analysis may be carried out during a normal 8-hour working day, leaving approximately one-half on an experienced operator's time for other duties.

On a typical day, the operator applies the new sample to be analyzed in the morning and starts the regeneration of the previous day's analytical column. During the morning hours, the Model K-8000 automatically performs the analysis for basic amino acids while regenerating the column used the previous day. Each afternoon, the operator prepares the instrument for the automatic all-night analysis for acidic and neutral amino acids and begins the equilibration of the column to be used on the following day. Facilities are also included to allow the Model K-8000 to automatically conclude an analysis and to shut itself down, eliminating the requirement for an operator to be present at the end of a run and thus allowing an additional analysis to be obtained each week.

The Model K-8000 is also equipped to perform the more complex and lengthy amino acid analysis of physiological fluids. Provisions have been included in the instrument which permit its use for the operation of preparative columns including stream-splitting with subsequent fraction collecting and simultaneous ninhydrin analysis. The Analyzer can also be used with scintillation counting accessory equipment with or without simultaneous ninhydrin analysis.

<u>Amino acids</u>

Amino acids by definition are organic compounds containing at least one carboxyl (acid) group and one amino (basic) group. They are the basic constituents of all proteins; the complete hydrolysis of a simple protein results in a mixture of amino acids. The amino acids are acid, neutral, or basic in reaction, as they possess respectively an overplus of carboxyl groups, an equality of carboxyl and amino groups, or an overplus of amino groups. The monoaminomonocarboxylic acids (such as glycine, alanine, valine, isoleucine, and leucine),

together with hydroxyamino acids (such as serine and threonine), sulfur-containing amino acids (such as cystine and methionine), aromatic amino acids (such as phenylalanine and tyrosine), and pyrrolidyl amino acids (such as proline) can conveniently be called "neutral amino acids".

Aspartic acid and glumatic acid each contain an extra carboxyl group and are therefore termed "acidic amino acids". Histidine, arginine, tryptophan, and lysine each contain an extra basic group (not necessarily an amino group) and are therefore termed "basic amino acids".

The Model K-8000 analyzes not only for amino acids, but small peptides, ammonia, urea, creatinine, amines, and other compounds with a ninhydrin reactive amino or imino group. Up to sixty compounds with which the analyst is principally concerned in the analysis of physiological fluids can be identified and measured.

Ion exchange chromatography

The Amino Acid Analyzer effects the separation of the amino acids in a sample by displacing them, in sequence, from a column of ion exchange resins. When an amino acid is placed on a column of the sodium salt of a polysulfonic resin, ion exchange takes place. Polysulfonic resin is a cation exchanger having negatively charged sulfonic acid groups; the amino acid molecule is attracted to the resin primarily through ionic forces by means of its positively charged amino group. This is a reversible reaction and equilibrium takes place. The amount of a given quantity of an amino acid which is bound to the ion exchange resin relative to that remaining in solution at equilibrium and under a given set of conditions is usually expressed as a distribution coefficient, K, and the magnitude of this coefficient depends on the structure of the individual amino acid.

As the amino acid still in solution filters down through the resin, the balance of equilibrium at the top of the column is destroyed, resulting in that portion of the resin releasing more of the amino acid molecules to restore the balance. As the amino acid solution travels down the column, the balance of equilibrium at each point is first established, then destroyed. The resin particle size must be sufficiently small, relative to the flow rate of the eluting buffer, to allow equilibrium conditions to prevail. When this is the case, the rate at which the zone of each individual amino acid moves down the column depends upon the distribution coefficient, K. This overall separation of the components in the sample is dependent on the chemical composition, resin particle size and resin pore size (degree of cross-linkage) of the resin, the diameter and length of the packed column, the charge and side group of the amino acid, the pH, ionic strength and rate of flow of the eluting buffer and the temperature of operation. Provided the capacity of the resin is not exceeded, each amino acid in the sample moves down the column in an individual

and independent zone. With the appropriate control of the foregoing variables, conditions are established which allow each amino acid to be separated from each other amino acid by the time it emerges from the column.

The basic amino acids have the strongest affinities for the resin while the acidic amino and hydroxyamino acids have the weakest. The ionic bond strengths of the remaining neutral acids lie between these, with the aromatic amino acids having the strongest of the neutral amino acids.

If the process of separation were to be limited to the flow of buffers through the columns by gravity or moderate air pressure, the duration of the period required for complete analysis would be unwieldy. In the Analyzer, buffers are forced through the columns by positive displacement pumps working at several atmospheres pressure to allow a complete protein analysis to be carried out in 22 hours. The amino-acid-containing sample is chromatographed on the resin columns with acidic sodium citrate buffers. In the analysis of protein hydrolyzates, two buffers are used with automatic change from the first to the second (which has a higher pH) midway through the analysis of the neutral and acidic amino acids. A third buffer with higher pH and higher ionic strength is used with a second column for the analysis of the basic components in the sample.

Color development and photometric determination

A number of polycarbonyl compounds undergo extensive reaction with amino acids. The most studied of these reagents, which lead to the formation of a colored compound, is ninhydrin, triketohydrindene hydrate. Ninhydrin undergoes an oxidation-reduction reaction with free amino groups, oxidatively deaminating them to carbonyl groups and ammonia. The reduced form of the ninhydrin couples with the ammonia and the residual ninhydrin to give rise to a blue-violet dye, diketohydrindylidene-diketohydrindamine (DYDA). The color formed from the reaction with the amino acids proline and hydroxyproline is yellow. The ninhydrin color reaction is positive for all free amino groups, whether in amino acids, peptides, or proteins.

The Amino Acid Analyzer makes use of this reaction to make a quantitative colorimetric analysis of each amino acid. By maintaining constant environmental factors, the color formation can be made proportional to only the quantity of the amino acid present. In a colorimeter containing three photometer units each consisting of a light source, a lens, an interference filter, a slit, the cuvette and a photoelectric cell, an electrical current is generated which is proportional to the color density of the effluent-ninhydrin mixture.

This electrical current is then used to drive a conventional multipoint recorder which plots the results of the analysis as absorbance versus time. As the DYDA from each amino acid passes

through the colorimeter, light to the photoelectric cell is reduced, resulting in a reduction of electrical output and a movement of the recorder pen. Three multipoint curves are plotted simultaneously consisting of a series of peaks, each peak corresponding to a specific amino acid.

APPENDIX F

Calculations and Chromatogram Interpretation[3]

The recorder chart travels at the rate of 3 inches per hour and is marked along its length with a light line every 0.1 inch and a heavy line every ½ inch. At the column elution rate of 30 ml/hr, each light line along the chart is equivalent to 1.0 ml elution volume. The chart is calibrated across in absorbance on a logarithmic scale from zero to infinity. The recorder prints a dot every 5 seconds; a dot on each of the three printed curves is thus printed every 15 seconds. On each of the three curves, every fourth dot is black, this pattern assisting in the integration of the area under each peak. Thus on each curve and for each 1.0 ml effluent volume (i.e., each 0.1 inch) eight dots are printed, two of them being black.

The amount of each component amino acid in a sample analyzed by the Amino Acid Analyzer is determined by measuring the area enclosed by its corresponding peak on the chromatogram. The height-times-width (HW) method of integration is rapid, satisfactorily accurate, and hence is used for the integration of the majority of the peaks.

A. Integration by height-times-width method

In using the HW method, the peak is multiplied by the width which is measured at half the height. The height of the peak is easily determined from the recorder chart and since the chart scale is logarithmic, the proportional accuracy with which a height value can be read is about the same regardless of the magnitude. The width of a peak is measured in terms of time by counting the number of dots printed above the half-height of the peak. The counting of the dots is facilitated by the fact that every fourth dot of each of the three curves is black.

After an Analyzer has been installed and is functioning properly, it is "standardized" by analyzing aliquots of Phoenix Amino Acid Calibration Mixture. From the standardization runs, an HW constant (C_{HW}) is calculated for each individual amino acid. These

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[3] From Phoenix Instruction Manual, Phoenix Precision Instrument Company, Philadelphia 40, Pensylvania.

constants are then used for the calculation of unknown quantities of components in other samples. As part of the initial standardization, the suppressed 570 mu conversion constant and the absorbance conversion constant is also determined. The former of these relates the peak areas under the normal 570 mu curve and the suppressed 570 mu curve and is used when suppressed 570 mu peaks are integrated. The absorbance conversion constant is used whenever integration by the absorbance method is carried out.

The data derived from the chart recording, the results of integrations, and further calculations are recorded on the Data Sheet. The 440 mu curve (brown) is used for proline and hydroxyproline. The normal 570 mu curve (red, normally the tallest) is used for all other peaks whose height does not exceed the absorbance value of 1.40. For any peaks whose height on the normal 570 mu curve exceeds 1.40, the suppressed 570 mu curve (green) is used. In the latter case, areas integrated under the suppressed 570 mu curve are converted to equivalent normal 570 mu areas by using as a factor the suppressed 570 mu conversion constant.

To integrate the area under a peak by the HW method, the procedure is as follows:

1. Read the base line to 0.001 absorbance units, and record the value in the Base Line column. This value is positive if the base line is above zero absorbance or negative if it is below zero. If the curve permits a reading of the base line at both sides of the peak, the average is used. If several peaks follow in close proximity, without the curve returning to a base line, and if there is a difference in the base line before and after the group of peaks, draw a straight line connecting the base lines and read the base line values for each peak concerned at the position of maximum height of the peak. For valine, which emerges just after the breakthrough of the pH 4.25 buffer, the base line following the peak should be taken.

2. Read and record in the Height column the height of the peak on the chart to 0.001 absorbance units for values below 1.00 absorbance and to 0.01 absorbance units for values above 1.00 absorbance. Take the reading at the top dot or dots of the peak.

3. The net height is obtained by subtracting algebraically the base line value from the height reading. Record this value in the Net Height column.

4. The half-height is determined by taking half of the net height value and adding algebraically to it the base line reading. Record this value in the Half Height column.

5. With fine pencil lines, mark the half-height level on the ascending and descending sides of the peak.

Data Sheet

Representative data for the calculation of the basic amino acids are illustrated.

Amino acid	Base line O.D.	Height O.D.	H net height O.D.	Half height on chart O.D.	W width Dots	H x W	u moles $\frac{H \times W}{C}$	mg amino acid per gm N
Lysine	0.007	0.910	0.903	0.459	17.4	15.71	0.73	463
Histidine	0.007	0.231	0.224	0.119	20.7	4.64	0.25	168
Ammonia	0.008	1.000	0.992	0.504	30.0	29.76	1.69	125
Arginine	0.008	0.370	0.362	0.189	37.0	13.39	0.71	537
$CySO_2H$								
Aspartic acid								
$MeSO_2$								
Threonine								
Serine								
Glutamic acid								
Proline								
Glycine								
Alanine								
Half cystine								
Valine								
Methionine								
Isoleucine								
Leucine								
Tyrosine								
Phenylalanine								

6. Starting from the half-height mark on the ascending side of the peak, and skipping the first black dot, count the number of black dots around the peak to the half-height mark on the descending side. Multiply this count by four.

7. Add to this figure the number of individual dots (of the same color) between the half-height mark and the first black dots on the ascending and descending sides of the peak. The first black dot on the ascending side should be included in this counting.

8. Finally, estimate the distance in tenths of dot separation between the half-height mark and the first individual dot up the curve. Repeat on the opposite side. Add both these estimations to the sum obtained in step 7. This is the width (W) in dots (or seconds/15) of the peak. Record this figure in the Width column.

9. To calculate the area of the peak, multiply the net height (H) in absorbance units from step 3 by the width (W) in dots from step 8 to obtain H x W. Record this value, to three significant figures, in the H x W column.

10. If the suppressed 570 mu curve has been used in integrating the peak, record the result at the left side of the H x W column. Multiply this figure by the suppressed 570 mu conversion constant and record the result on the right side of the same space. Separate the two values by a diagonal line. The latter converted H x W value is the one that is subsequently used either in calculating the HW constant or in calculating the unknown concentration.

B. Calculations of concentrations in unknown mixtures

After the completion of the standardization of the Amino Acid Analyzer and after having integrated the peaks on the chromatogram of an unknown sample as described in subsection A, the concentrations of each of the individual amino acids in the unknown are calculated as follows:

1. Divide the calculated H x W value for the unknown by the appropriate HW constant to give the concentration in u moles. Record these values in the Micromoles column of the Data Sheet. At the top of the column, note the date for the set of standard HW constants which were used in the calculations.

2. The empty columns may be used for noting the calculated concentration of components in terms of micromoles per unit of volume, weight or time such as per total sample, per mg, per day, etc.

C. Integration by the absorbance method

The total ninhydrin color in a given peak can be obtained by addition of the absorbance values read from the recorded curve. The

addition takes more time than the measurement of H and W, but is preferable for very small peaks such as the methionine sulfoxides, overlapping peaks such as 3-methylhistidine and anserine, or a very asymmetric peak. The determination of cystine in protein hydrolyzates is a common instance requiring the addition of absorbance readings. The presence of meso-cystine, formed as a result of racemization during hydrolysis leads to the appearance of a markedly asymmetric cystine peak that cannot be integrated by the H x W method. To integrate peaks such as those described above by the absorbance method proceed as follows:

1. For each peak, add the absorbance values read along the curve at each 1 ml interval, i.e., each light line.

2. Make a base-line correction by subtracting the average base-line reading multiplied by the number of values taken. The remainder is the area of the peak expressed as net total absorbance.

3. Convert the value from step 2 to micromoles of amino acid with the following equation:

$$u \text{ Moles} = \frac{NTA_u}{NTA_s}$$

NTA_u = Net total absorbance for unknown

NTA_s = " " " " standard